Journal of Chromatography, 588 (1991) 281–287
Elsevier Science Publishers B.V., Amsterdam

CHROM. 23 661

Gas chromatographic–mass spectrometric analysis of ginkgolides produced by *Ginkgo biloba* cell culture

Nathalie Chauret*

Department of Medicinal Chemistry, Merck Frosst Canada, C.P. 1005, Pointe-Claire-Dorval, Quebec H4R 4P8 (Canada)

Julie Carrier

Department of Chemical Engineering, McGill University, 3480 University Street, Montreal, Quebec H3A 2A7 (Canada)

Michael Mancini

Biotechnology Research Institute, National Research Council Canada, 6100 Royalmount Avenue, Montreal, Quebec H4P 2R2 (Canada)

Ronald Neufeld and Martin Weber

Department of Chemical Engineering, McGill University, 3480 University Street, Montreal, Quebec H3A 2A7 (Canada)

Jean Archambault

Biotechnology Research Institute, National Research Council Canada, 6100 Royalmount Avenue, Montreal, Quebec H4P 2R2 (Canada)

(First received May 13th, 1991; revised manuscript received July 29th, 1991)

ABSTRACT

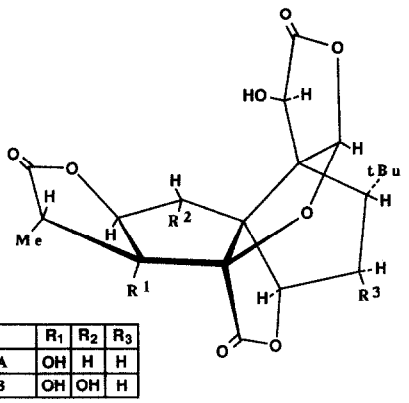
An analytical method was developed to confirm the production of ginkgolides by *Ginkgo biloba* cells cultured *in vitro*. Biomass samples were extracted for 16 h with acetone. This extract was loaded onto a silica gel column and purified by differential elution using a cyclohexane–ethyl acetate solvent system. The recoveries of ginkgolide A and ginkgolide B were × ei90% and × ei35%, respectively. Subsequently, this purified extract was trimethylsilylated for gas chromatographic separation of ginkgolide A and ginkgolide B. Flame-ionization detection was not selective enough for identification of ginkgolides A and B in the extracts. The ginkgolides were detected by coupling the gas chromatograph to a high-resolution mass spectrometer operated in the selected-ion monitoring mode. The concentration of ginkgolides A and B in culture extracts was determined by gas chromatography–mass spectrometry using deutero-trimethylsilylated ginkgolides as internal standards. This technique permitted detection of ginkgolides A and B at concentrations as low as 10^{-1} pmol/μl of injected purified extract.

INTRODUCTION

Ginkgolides are potent and specific platelet antagonist factors (PAF) that are presently undergoing clinical trial in Europe to treat asthma and allergy [1]. The synthesis of these phytochemicals (Fig. 1) is complex, making large-scale production difficult [2]. The main source of ginkgolides is the leaves of the slow-growing tree *Ginkgo biloba* L. [3].

Lobstein-Guth *et al.* [4] have developed an extensive purification method using liquid–liquid extraction and preparative chromatography to prepurify

0021-9673/91/$03.50



	R_1	R_2	R_3
GA	OH	H	H
GB	OH	OH	H

Fig. 1. Chemical structures of ginkgolides.

ginkgolides obtained from leaf extracts and have used high-performance liquid chromatography (HPLC) and ultraviolet (UV) detection at 220 nm for analysis. The low UV absorption coefficient of ginkgolides resulted in a detection limit of ~ 70 nmol [4]. Van Beek *et al.* [5] reduced this detection limit to 7 nmol using refractive index detection after HPLC separation, which is similar to that obtained by Tallevi and Kurz [6] using thin-layer chromatography (TLC) and UV visualisation of ginkgolides obtained from commercial plant extracts.

G. biloba cell culture is an alternative to agricultural supply of ginkgolides. However, Nakanishi and Habaguchi [7] could not detect ginkgolides in cultured cells using HPLC. These results suggested that cultured cells were not producing ginkgolides or were producing them at concentrations lower than the detection limit of their analytical technique.

In order to confirm the presence of ginkgolides in cultured *G. biloba* cells, a detection method that is more sensitive and selective than those used previously is required. Mass spectrometry (MS) was found to be suitable for this purpose. The analysis of ginkgolides by MS has not been extensively documented. Nakanishi [8] seems to be the only one who has reported on the lactone structure of ginkgolide A (GA) derivatized as a dimethyl ether using MS.

First experiments conducted in our laboratory have shown the usefulness of gas chromatography (GC) combined with electron-impact (EI) MS for identification and quantification of trimethylsylilated GA in crude cultured cell extracts [9]. These analyses, which were performed on resinous crude extracts, resulted in numerous GC–MS problems (contamination of the injector, column and MS source). This method could not be used routinely. This work describes a procedure to purify biomass extracts using a silica gel column and GC–MS protocols for proper characterization and analysis of ginkgolides A and B.

EXPERIMENTAL

Chemicals

Ginkgolide standards A and B were kindly provided by Dr. P. Braquet (Institut Henri Beaufour, Les Ulis, France). Stock solutions of these standards (~ 120 pmol μl^{-1}) were prepared monthly in methanol and stored at 4°C. All solvents (methanol, acetone, ethyl acetate and cyclohexane) were HPLC grade (Caledon, Georgetown, Canada). *G. biloba* cells, cultured as described elsewhere [9,10], were the experimental source of ginkgolides.

Sample preparation

Lyophilized *G. biloba* cells (20 g) were extracted with acetone (1 l) for 16 h at ambient temperature with gentle mixing. The suspension was filtered (0.45-μm HVLP filter, Millipore, Bedford, MA, USA) by vacuum. The acetone extract was evaporated to dryness on a Rotavap (Brinkman, Westbury, NY, USA). The residue was redissolved in ~ 5 ml of cyclohexane–ethyl acetate (50:50, v/v), yielding a dark-brown syrup (fraction I).

For GC–flame-ionization detection (FID) analysis, all the volume of fraction I was enriched with GA and GB (~ 60 nmol) prior to purification (fraction IE). For GC–MS analysis, fraction I was divided in two equal volumes (fractions Ia and Ib). Before purification on silica gel, fraction Ia was enriched with 5 nmol of GA and 5 nmol of GB (fraction IaE). No ginkgolide was added to fraction Ib.

Biomass extract purification

The purification of fraction IE was done using a silica gel column (Kieselgel, 230–400 mesh, Merck, Darmstadt, Germany) as suggested by Lobstein-Guth *et al.* [4] for purification of leaf extracts. The column (11 cm × 2.5 cm I.D.) was prepared from a slurry of silica gel (20 g) in ~ 75 ml of cyclohexane–

ethyl acetate (50:50, v/v). The column was conditioned with ~ 200 ml of cyclohexane–ethyl acetate (50:50, v/v) at a flow-rate of ~ 1 ml min^{-1} 16–24 h before use. Fraction IE was loaded on the column, which was washed with 60 ml of cyclohexane–ethyl acetate (50:50, v/v) at ~ 1 ml min^{-1}. This first fraction (green) was discarded. Thereafter, the column was eluted with 80 ml of cyclohexane–ethyl acetate (20:80, v/v) at ~ 1 ml min^{-1}. This second fraction (yellow) was collected and evaporated to ~ 5 ml (fraction IIE). Fractions IaE and Ib for GC–MS analysis were purified simultaneously with two silica columns similarly. The resulting fractions were designated IIaE and IIb.

Derivatization

Purified fractions (~ 5 ml) were transferred to a test vial, evaporated to dryness under a stream of nitrogen at 38°C and trimethylsilylated by adding 100 μl of N,O-bis(trimethylsilyl)acetamide (BSA) (Trisil BSA DMF, Pierce Chemicals, Rockford, IL, USA). This mixture was vortexed and heated for 1 h at 75°C.

Standards for GC–FID analysis were prepared by combining 100-μl volumes of GA and GB stock solutions, which were evaporated to dryness and trimethylsilylated wtih 200 μl of BSA as above. This yielded a standard solution of ~ 60 pmol μl^{-1} GA and GB.

For GC–MS standards, appropriate volumes of GA and GB stock solutions were combined, evaporated to dryness and trimethylsilylated with 100 μl of BSA to yield five standards of concentrations of 0.25, 2.5, 25, 60 and 120 pmol μl^{-1} GA and GB.

GC analysis

Analyses by GC–FID were performed with a Perkin-Elmer Sigma 2000 gas chromatograph (Norwalk, CT, USA) equipped with a 30 m × 0.25 mm I.D. DB1 WCOT capillary column (J&W Scientific, Folsom, CA, USA). Helium was used as the carrier gas at a head pressure of 2 bar. The temperatures of the injector, column and detector were 295°C, 270°C and 345°C, respectively. Injections (2 μl) were made in the splitless mode. Retention times of GA and GB were 6.7 and 7.7 min, respectively.

Analyses by GC–MS were performed with a Varian 6000 gas chromatograph (Sunnyvale, CA, USA) equipped with the same capillary column as above and an on-column injector (J&W Scientific). The gas chromatograph was interfaced directly to a VG ZAB-HF mass spectrometer (Middlewich, UK). Helium was the carrier gas at a head pressure of 1 bar. The temperature was increased from 250°C to 275°C at a rate of 5°C min^{-1}, and then to 285°C at a rate of 1°C min^{-1}, and finally to 300°C at a rate of 15°C min^{-1}. This temperature was maintained for 5 min.

The MS conditions were: electron-impact ionization, −70 eV; trap current, 100 μA; source temperature, 275°C. All re-entrants and transfer lines were maintained at 250°C. Resolution was 7500 (10% valley definition). Chromatograms were acquired in the selected-ion monitoring (SIM) mode using accelerating voltage switching with a dwell time of 120 ms each and a 10-ms switching delay between the eight ions monitored with m/z ranging from 537 to 667. Perfluorokerosene (PFK) was used as a lock-mass compound.

Internal standards were required for quantification of GA and GB by GC–MS. The internal standards chosen were deutero-trimethylsilylated GA and GB, which were prepared by adding 100 μl of deutero-regisil (Regis, Morton Grove, IL, USA) and 250 μl of dimethylformamide (DMF; Regis) to 20 nmol of GA and 20 nmol of GB. This mixture was mixed vigorously and heated for 1 h at 75°C. A volume of 5 μl of deutero-trimethylsilylated GA and GB was added to derivatized fractions IIaE and IIb or to derivatized standards prior to GC–MS analysis. This mixture yielded final deutero-trimethylsilylated GA and GB concentrations of 3 pmol μl^{-1}. Quantitative analysis by GC–MS was done using the ratio of the peak area of known concentrations of derivatized GA and GB to that of deutero-derivatized GA and GB internal standards. These calibration curves were linear from 0 to 120 pmol μl^{-1} with correlation coefficients of ~0.99 for both GA and GB. They were used to assess the concentration of ginkgolides in fractions IIaE and IIb.

RESULTS AND DISCUSSION

GC–FID analysis

Derivatization reaction. Ginkgolides are non-volatile compounds and need to be derivatized to be made volatile and analysed by GC–FID. Derivatization using trimethylsilylating agent (BSA) was

chosen since a simple chemical reaction is involved. A typical procedure for derivatization with BSA recommends the use of 1200–2400 nmol of compound per 100 μl of BSA reagent [11]. In the present study, the quantity of BSA required to achieve complete trimethylsilylation of ginkgolides contained in purified biomass extracts needed to be determined. Since actual cultured biomass extracts were known to contain low quantities of ginkgolides (~ pmol g^{-1} dry weight) [9], this was done by adding different excess amounts of pure GA and GB (~7–60 nmol) to various purified fraction I mixtures not enriched with these compounds before purification. These mixtures were derivatized with a constant volume of BSA (100 μl) and analysed by GC–FID. The resulting GA and GB peak areas were divided by the amount of GA and GB added to the purified extracts. The resulting ratios were constant [125 mV s $nmol^{-1}$ for GA and 135 mV s $nmol^{-1}$ for GB, with a relative standard deviation (R.S.D.) of 10 mV s $nmol^{-1}$ calculated from four different assays], indicating that BSA was in excess and complete trimethylsilylation was achieved. Consequently, sample preparation required at most 100 μl of BSA per 60 nmol of ginkgolides in purified extracts.

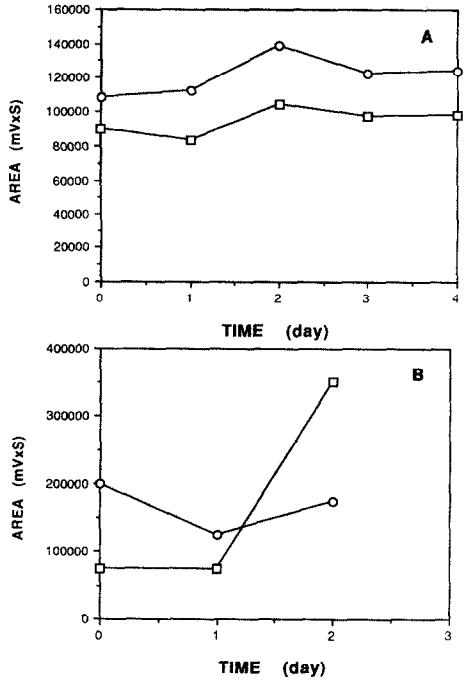


Fig. 2. Stability of trimethylsilylated derivatives (A) Pure ginkgolides. (B) Ginkgolides in enriched cell extracts. □ = GA; ○ = GB.

The chemical stability with time of trimethylsilylated GA and GB was evaluated. As shown in Fig. 2A, derivatized pure GA and GB were stable for at least four days. On the other hand, ginkgolides present in biomass extracts (fraction IIE) appeared to be degraded after one day, as indicated by significant differences in peak areas (Fig. 2B). This degradation may be attributed to hydrolysis since trimethylsilylated compounds are known to be moisture-sensitive [11] and/or appears to result in the formation other compound(s) interfering with ginkgolide detection. Consequently, purified biomass extracts needed to be analysed on the day of derivatization.

Purification procedure. Initially, the elution and recovery of pure ginkgolides from silica gel columns prepared as described above using a solvent system of 50:50 (v/v) cyclohexane–ethyl acetate, as suggested by Lobstein-Guth *et al.* [4], was evaluated. The rate of desorption of GA (~60 nmol) and GB (~60 nmol) was determined by collecting the effluent from the silica gel column in ~10-ml fractions which were analysed by GC–FID after derivatization. Recovery of GA was ~45%. However, GB, being more polar, was dispersed in many fractions, impairing its proper recovery. A more efficient scheme was devised using initially 60 ml of 50:50 (v/v) cyclohexane–ethyl acetate to separate hydrophobic products suspected to be present in actual extracts from ginkgolides. Thereafter, a 20:80 (v/v) cyclohexane–ethyl acetate solvent was used to elute GA and GB, which were recovered mainly between volumes of 65 and 120 ml with efficiencies of 90 ± 10% and 35 ± 10%, respectively (R.S.D. from three independent experiments). A more polar solvent (100% ethyl acetate) did not improve GB recovery.

Similarly, fraction IE purified on silica columns showed GA and GB to be eluted with 20:80 (v/v) cyclohexane–ethyl acetate at fractions between 70 and 130 ml. Fractions between 40 and 60 ml could not be analysed by GC–FID since a precipitate formed upon derivatization. Fig. 3, presenting the GC–FID chromatogram of derivatized fraction IIE, shows many organic compounds eluting together with GA and GB, rendering difficult their identification and quantification.

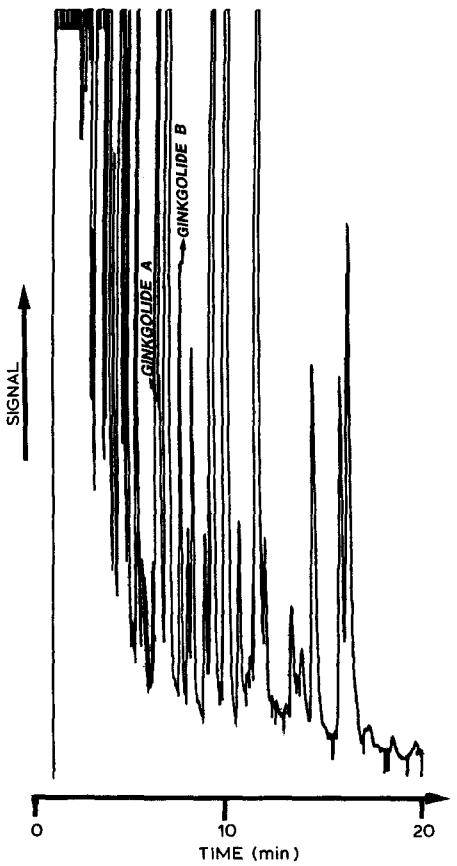


Fig. 3. GC–FID profile of trimethylsilylated ginkgolides in fraction IIE.

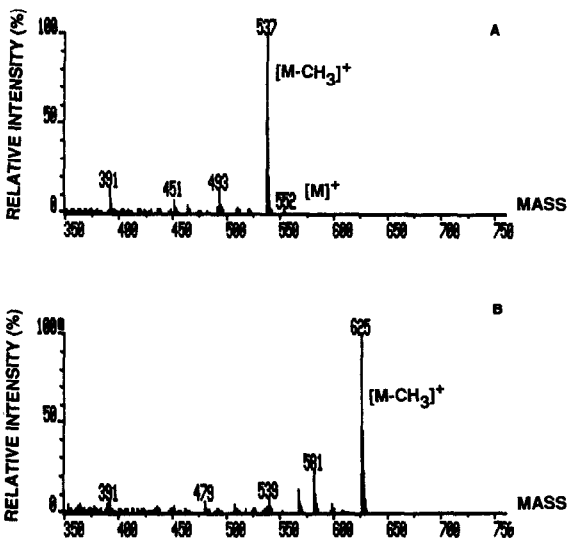


Fig. 4. Partial mass spectrum of trimethylsilylated ginkgolides measured at −70 eV EI. (A) GA. (B) GB.

The purification procedure converted resinous fraction I into a clear, injectable extract but was not sufficient to permit GC–FID analysis of GA and GB in the purified biomass extract. A more selective and sensitive detection technique, such as GC–MS, was preferred over further purification steps since cultured *G. biloba* cells were suspected to yield low concentrations of GA and GB.

GC–MS analysis

MS analysis of GA and GB. Derivatized GA and GB standards were analysed by GC–MS to evaluate their fragmentation pattern in the experimental conditions used. Fig. 4A presents the partial mass spectrum of trimethylsilylated GA, which displayed a weak molecular ion M^+ with *m/z* 552.221 in the 70-eV electron-impact (EI) spectrum. The major fragment with *m/z* 537.197 resulted from the loss of a methyl group. Trimethylsilylated GB displayed no molecular ion M^+ with *m/z* 640.256, as shown in Fig. 4B. The major fragment ion with *m/z* 625.232 resulted, as for derivatized GA, from the loss of a methyl group. The fragmentation patterns of trimethylsilylated GA and GB were very similar, consisting mainly of losses of 15, 59 and 101 a.m.u. from their respective molecular ion M^+. Deuterated trimethylsilylated GA and GB displayed weak molecular ions with *m/z* 570.334 and *m/z* 667.4245. Major ions resulting from the loss of a deuterated methyl group were observed at *m/z* 552.298 and *m/z* 649.383 for deuterated GA and GB, respectively.

The intense fragments resulting from the loss of methyl groups from the molecular ions were chosen for quantitative analysis in the SIM mode. Fig. 5A and B presents the mass chromatograms of non-deuterated and deuterated trimethylsilylated GA with retention times of 11.0 and 10.8 min, respectively. Mass chromatograms of non-deuterated and deuterated trimethylsilylated GB with retention times of 12.5 and 12.2 min, respectively, are shown in Fig. 5C and D.

The response factors [RF = (signal of the analyte × amount of the internal standard)/(signal of internal standard × amount of analyte)] were

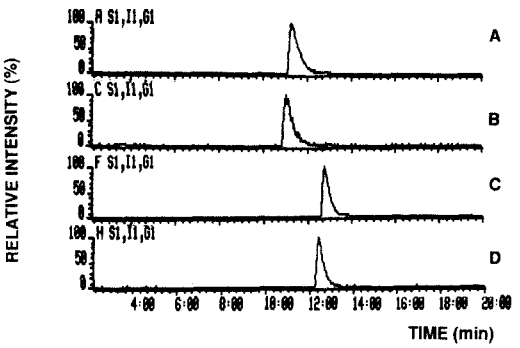


Fig. 5. Mass chromatograms of non-deuterated and deuterated trimethylsilylated ginkgolide standards. Each trace was normalized to the largest peak within the time window. (A) Trimethylsilylated GA: $[M-CH_3]^+$ at *m/z* 537.197. (B) Deutero-trimethylsilylated GA: $[M-C^2H_3]^+$ at *m/z* 552.221. (C) Trimethylsilylated GB: $[M-CH_3]^+$ at *m/z* 625.232. (D) Deutero-trimethylsilylated GB: $[M-C^2H_3]^+$ at *m/z* 649.383.

1.1 ± 0.1 and 1.0 ± 0.1 for GA and GB, respectively. These RF values near unity indicate that non-deuterated and deuterated trimethylsilylated ginkgolides behave similarly, satisfying an important criterion for selecting a good internal standard.

Analysis of cultured G. biloba cell extracts. Biomass extracts were purified and analysed by GC–MS. Fig. 6 presents typical mass chromatograms of a purified cell extract (fraction IIb) supplemented with internal standards. The retention times of fragment ions *m/z* 537.197 and *m/z* 552.221 were 10.7 and 10.6 min, respectively, which are similar to those of non- and deuterated derivatized GA standard. The retention times of ions *m/z* 625.232 and 649.383 were 12.0 and 11.7 min, respectively, in accordance with the retention times found for GB standards. Slight differences in retention times of GA and GB from standards and samples can be attributed to variations in the experimental conditions.

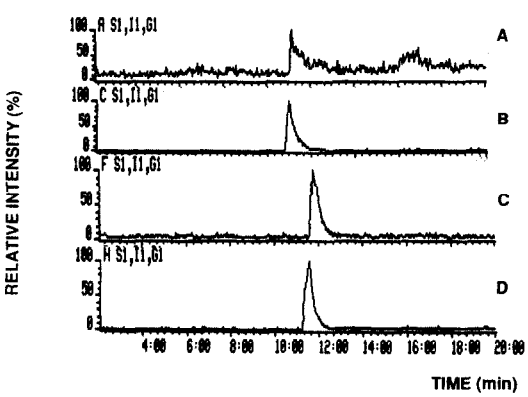


Fig. 6. Mass chromatograms of non-deuterated and deuterated trimethylsilylated ginkgolides in fraction IIb. Each trace was normalized to the largest peak within the time window. (A) Trimethylsilylated GA: $[M-CH_3]^+$ at *m/z* 537.197. (B) Deutero-trimethylsilylated GA: $[M-C^2H_3]^+$ at *m/z* 552.221. (C) Trimethylsilylated GB: $[M-CH_3]^+$ at *m/z* 625.232. (D) Deutero-trimethylsilylated GB: $[M-C^2H_3]^+$ at *m/z* 649.383.

The addition of deutero-trimethylsilylated ginkgolides to samples allowed normalization of the results of the GC–MS analysis and evaluation of GA and GB concentration in fraction IIb. As shown in Table I, GC coupled to electron-impact high-resolution MS allowed the quantitative determination of GA and GB in the range of 10^{-1} pmol μl^{-1} purified extracts. The addition of the internal standards prior to GC–MS prevented evaluation of the recovery efficiency of the whole procedure (purification and chromatographic separation). The use of a different internal standard before the purification of fraction I may have been a better method of evaluating the efficiency of recovery of the whole procedure. However, no chemical product that behaved to some extent like ginkgolides was found. The efficiency of the purification step was estimated by comparing results obtained from the analysis of enriched fraction II (fraction IIaE) and non-enriched fraction II (fraction IIb). As reported in Table I, recovery of GA and GB were 85 ± 10% and 30 ± 13%, respectively. These values are in the same range as those determined by GC–FID for pure GA and GB.

Ginkgolide yields of various *G. biloba* cell samples are presented in Table I. These figures were normalized by taking into account the recovery efficiency of the purification procedure. These low yields confirm the non-suitability of HPLC and TLC methods with refractive index or UV detection for the determination of ginkgolides produced by *G. biloba* cultured cells at this stage of development of this biological system. The procedures developed herein confirmed that GA and GB are produced by cell cultures, although in the pmol g^{-1} dry weight range presently. This method is used in our laboratory to screen different cell lines and culture con-

TABLE I

GC–MS ANALYSIS OF GA AND GB IN FOUR *G. BILOBA* CULTURED CELL SAMPLES

Sample No.	Concentration in prepurified extract (fraction IIb) (pmol μl^{-1})		Recovery from the extraction procedure [a] (%)		Concentration in cultured cells[b] (pmol g^{-1} dry weight)	
	GA	GB	GA	GB	GA	GB
1	0.6	0.2	97	32	6.4	5.8
2	2.9	0.5	81	22	56	35
3	2.7	0.9	75	23	56	52
4	0.4	0.7	85	40	5.3	17

[a] Recovery of GA and GB from the extraction procedure was evaluated using the enriched fraction (fraction IIa).
[b] Concentration in cells reported on a dry weight basis are normalized by taking into consideration the efficiency of the recovery of GA and GB from the extraction procedure.

ditions to optimize production of ginkgolides by *G. biloba* cell cultures.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. Ian Reid for useful editorial comments. D. J. Carrier was the recipient of an F.C.A.R. scholarship.

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