Invited lecture/Research

A Multiplex GC-MS/MS Analysis for the Quantitative Monitoring of Bilobalide, Ginkgolides and Ginkgotoxin in *Ginkgo biloba*-Derived Products and Biomaterials

Árva Zsolt [1,2], Barbulova Ani[1], Fiume Immacolata[1], Moubarak Maneea[1,3], Pocsfalvi Gabriella[1]

1. Laboratory of Extracellular Vesicles and Mass Spectrometry, Institute of Biosciences and BioResources, Italian National Research Council, Naples, Italy
2. Faculty of Science and Technology, University of Debrecen, Hungary
3. Faculty of Agriculture, Damanhour University, Damanhour, Egypt

* Correspondence: Gabriella Pocsfalvi; gabriella.pocsfalvi@ibbr.cnr.it

Abstract:
The bioactivity *Ginkgo biloba*-derived extracts and other preparations is attributed to the presence of secondary metabolites, especially terpene trilactones (ginkgolides and bilobalide), flavonoids but also toxic constituents, like ginkgotoxin. In this study, we set up a multiplex method using a gas chromatography coupled with tandem mass spectrometry (GC-MS/MS) for the simultaneous quantitative analysis of six of these characteristic metabolites, namely ginkgolide A, B, C, J, bilobalide and ginkgotoxin. Parameters were set up and optimized for multiple reaction monitoring (MRM) to allow the sensitive and selective monitoring of specific collision induced dissociation transitions for each analyte. The method was applied to quantify and compare the above ingredients after derivatization in the methanol extracts of a commercial supplement, ginkgo nuts, ginkgo cells from suspension and callus cultures, and microvesicles (MVs) and nanovesicles (NVs) isolated from ginkgo seeds. As a result of the MRM analysis, the commercial supplement contained 7,77% terpene lactones per tablet. The seed samples contained 50 parts per million (ppm) ginkgolide A and 14 ppm ginkgolide B, but ginkgolide C and J were below the limit of quantitation. Ginkgo biloba cells from in vitro cultures, MVs and NVs contained considerably less bilobalide and ginkgolides than the seed and the commercial supplement.

Keywords: *Ginkgo biloba***;** extracellular vesicles; GC-MS/MS; derivatization; quantitative analysis, multiple reaction monitoring, ginkgolides, bilobalides, ginkgotoxin

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1. Introduction

Ginkgo biloba L., the only survivor of genius ginkgo is a living fossils revered for its longevity and elegance of its leaves (Chi et al., 2020). Different parts of ginkgo tree such as leaves, seeds and roots have been used in traditional Chinese medicine for thousands of years. A standardized extract of ginkgo biloba (GB) dried leaf, Egb 761 containing 24% Ginkgo Flavone Glycosides and 6% Terpene lactones is among the most studied and most popular herbal supplements today (Nor-E-Tabassum, 2022). Egb 761 represents the only herbal alternative to synthetic antidementia drugs in the therapy of Alzheimer's disease and cognitive decline (Nor-E-Tabassum, 2022). Sora *et al* developed and validated a liquid chromatography tandem mass spectrometry (LC–MS/MS) assay of terpene trilactones in (GB) extracts and pharmaceutical formulations through standard addition method and found bilobalide content 2.8-3.2 mg and ginkgolides (A, B, C) content 2.9-3.2 mg (Sora et al., 2009). Gas chromatography - mass spectrometry (GC-MS) has also been successfully applied to quantify ginkgolides in ginkgo dietary supplements (Deng and Zito, 2003).

The edible seeds of GB are also used in traditional Chinese medicine for different purposes, including the treatment of senility, asthma, bronchitis, and kidney and bladder disorders. GB seeds known to contain toxic compounds like ginkgotoxin (N-methyl pyridoxine), an analogue of Vitamin B6, that raised concern about its use (Boateng and Yang, 2022). LC-MS and LC-MS/MS methods have been developed for the identification and quantitation of ginkgotoxin (Scott et al., 2000) and determined 174 ppm ginkotoxin in seed extracts.
In this study, our goal was to develop a multiplex analytical method based on gas chromatography-tandem mass spectrometry (GC-MS/MS) for the simultaneous quantitation of five terpene lactones (ginkgolide A, B, C, J and Bilobalide) and ginkgotoxin (**Figure 1**).

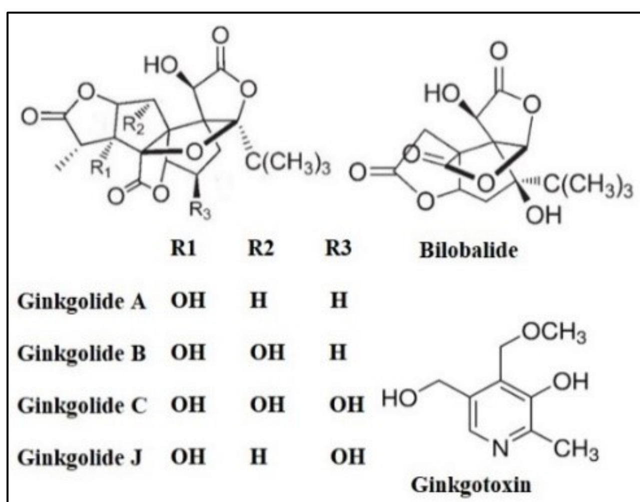

Figure 1. Chemical structures of ginkgolides A, B, C, J, bilobalide and ginkgotoxin.

The developed method was used to investigate the quantities of these primary ginkgo specific ingredients in the methanol extract of ginkgo nuts, ginkgo cells from suspension and callus cultures, micro- and nanovesicles isolated from seeds as well as a commercial supplement (**Figure 2**). While there have been LC-MS/MS based studies on terpene lactones that investigates commercial pharmaceutical products containing standardized extracts (Sora et al., 2009; Scott et al., 2000), to the best of our knowledge there is no GC-MS/MS method available today for the simultaneous analysis of terpene lactones and ginkgotoxin.

Figure 2. Samples analysed; A) Gloryfeel ginkgo biloba supplement prepared from ginkgo leaves, B) ginkgo seeds C) embryo, callus and cell suspension cultures.

2. Materials and methods

2.1. Sample preparation and standards

GB dietary supplement was from Gloryfeel BmbH (Hamburg, Germany). Tablet (274.05 mg) was pulverized in a mortar and sample (235.6 mg) was extracted in 5 mL methanol (Romil MS grade) for 30 minutes under orbital shaking at room temperature. Samples were centrifuged at 14000 x g at 26 °C for 20 minutes two times.

GB seeds were collected in Naples Piscinola in November, 2023. Seed shell and inner layer were removed and 5 samples containing 5 seeds were dried for 72 h at 40 °C. Samples were pulverized in a mortar and 50 mg were extracted in 600 µL methanol.

Callus and cell suspension cultures were established using GB embryos as starting material. The seeds were surface sterilized with sodium hypochlorite and subsequently, viable GB embryos were isolated and put for callus induction on Gamborg B5 growth medium (Duchefa Biochemie), supplemented with 2 mg/L of 2,4-dichlorophenoxyacetic acid (2,4D) and 2 mg/L of naphthyl acetic acid (NAA). White and friable callus cultures were obtained after 8 weeks of cultivation in dark conditions and were maintained by subculturing every 4 weeks. To initiate cell suspension cultures, 5 g of callus were resuspended in 50 mL of the same growth medium and incubated at 26 °C in dark conditions under constant orbital stirring (110 rpm). The cultures were maintained by subculturing every 7 days. To prepare the samples, 40-45 days old callus cultures and 7 days old suspension cultures were used. Samples were homogenized in methanol using a Tissuelyser (Qiagen) at 30 Hz, 5 x 1 minutes.

Microvesicles (MVs) and nanovesicles (NVs) were isolated from the homogenate of GB seeds using the differential ultracentrifugation method (Bokka et al., 2020). Protein concentration was measured by Qubit assay (Invitrogen). 300 µg MVs and NVs expressed in protein quantity were extracted in 300 µL methanol. The samples were homogenized in methanol using a Tissuelyser (Qiagen) at 30 Hz, 5 x 1 minutes.

Five replicates were prepared of the suspension and callus cultures, seeds and tablet, and triplicates for MVs and NVs samples. All methanol extracts were centrifuged at 15000 x g for 20 min. 600 ng D-Mannitol-$^{13}C6$ (Cambridge Isotope Laboratories, Canada) was added as internal standard (IS). Samples were vacuum dried and then further dried under nitrogen flow. Derivatization was performed using 50 µL silylation derivatization reagent, (N,O-bis[trimethylsilyltrifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) (Sigma Aldrich) and 35 µL pyridine (Merck) at 70 °C for 1 hour at 500 rpm agitation in a thermomixer.

The calibration solutions were prepared in the following ranges: ginkgolide A, B, C and J from 0.05 µg to 50 µg; bilobalide from 0.05 to 30 µg and ginkgotoxin from 0.01 to 20 µg in 85 µL. The quantity of the IS was maintained at 600 ng in 85 µL in each standard solution.

2.2. GC-MS/MS measurement parameters

GC-MS/MS analyses were performed using a Thermo Scientific Trace 1300 GC coupled to a TSQ 8000 Duo mass spectrometer equipped with an electron ionization (EI) ion source and a triple quadrupole (QqQ) ion analyzer. Separation was performed using a TG-SQC 30 m, 0.25 mm x 0.25 µm capillary column. GC parameters were as follows: measurement time: 35 minutes, column flow: 1.2 mL/min (helium), injection volume: 1.0 µL, injection type: splitless, inlet temperature 230 °C. MS parameters; transfer line temperature: 280 °C, ion source temperature: 250 °C. GC temperature ramp; initial temperature: 70 °C hold for 1 min, ramp rate 1: 10 °C/min to 280 °C, ramp rate 2: 6 °C/min to 313 °C, ramp rate 3: 0,6 °C/min to 314 °C, ramp rate 4: 6 °C/min to 316 °C, ramp rate 5: 2,4 °C/min to 318 °C, ramp rate 6: 6 °C/min to 320 °C hold for 2 min.

Dionex Chromeleon 7 Chromatographic Data System version 7.2.10 (Thermo Scientific Fisher, USA) was used for data acquisition and processing. The tentative identification of the compounds was based on computer matching with the National Institute of Standards and Technology (NIST) library. Collision induced dissociation (CID) was performed using Argon as collision gas. Two ion transmissions of the single analytes were selected by using the Thermo Scientific AutoSRM application run under Chromeleon 7 software package. Concentrations of the analytes in the samples were calculated using calibration curves values corrected against the internal standard.

3. Results and Discussion

Full scan MS-analysis using chemical derivatization to make the compounds volatile provides an unbiased identification of metabolites in plant extracts (Fiehn, 2016). In this study, after silylation derivatization we have performed full scan GC-MS analyses of the methanol extracts of the following ginkgo biloba-derived samples: i) seed, ii) callus iii) cells from suspension culture, iv) microvesicles isolated from GB seeds, and v) nanovesicles isolated form GB seeds. Compounds that were identified in at least 3 samples were considered. Appendix A shows the compounds tentatively identified in each sample. Mostly primer metabolites, i.e. aminoacids, lipids, organic acids, aldehydes, ketones and alcohols and sugars could be identified in this analysis. 100 compounds were identified in the GB seed extract, 60 in callus culture, 68 in cell suspension derived cells, 35 in microvesicles and 39 in nanovesicles derived from GB seeds. Venn diagrams compare the presence of different metabolites in seed and seed-derived micro- and nanovesicles samples (Figure 3A) and in seed and seed explant-derived callus and suspension cultures (Figure 3B). Only 10 compounds were commonly present in MVs, NVs and seed extracts, 7 compounds in NVs and seed, 8 compounds in MVs and seed, and 13 compounds in MVs and NVs (Figure 3A). 30 compounds were commonly identified in callus, suspension cultures and seed, 3 compounds in callus and seed, 6 compounds in suspension cultures and seed, and 19 compounds were commonly identified in callus and suspension cultures (Figure 3B). Interestingly, only two secondary metabolites, the flavonoid monomeric unit containing catechin (2R-trans) and epigallocatechin, were detected in callus and suspension cultures. Characteristic GB metabolites, like bilobalide, ginkgolides or ginkgotoxin could not be identified through the full scan GC-MS analysis in seed and seed derived cells cultured in vitro or seed-derived micro and nanovesicles.

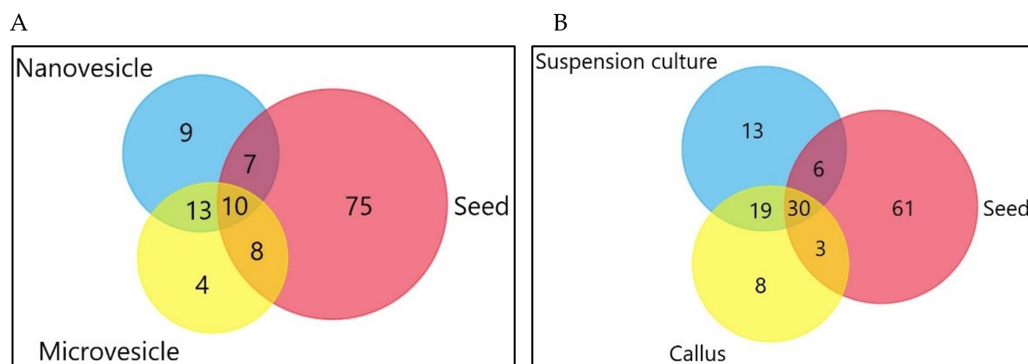

Figure 3. Venn diagram of A: the metabolites identified in MVs, NVs and ginkgo biloba seeds and B: callus, suspension cultures and ginkgo biloba seeds (lists of compounds are reported in **Appendix A**).

To setup the GC-MS/MS targeted analysis method, we have determined and optimized two characteristic precursor-to-product ion transitions for the internal standard mannitol ^{13}C6 and six ginkgo biloba secondary metabolites, i.e. ginkgotoxin, bilobalide and ginkgolides A, B, C and J. These are reported in **Table 1**. Collision energy (CE) for each transition was optimized using the Chromeleon AutoSRM software and the optimal CE values are reported in **Table 1**.

Table 1. Precursor / product ion's *m*/*z* as well as collision energy values.

Compound	m/z Precursor/Product Ion	Collision Energy (eV)
ginkgotoxin	280.1 / 206.1	10
	295.1 / 280.1	10
D-mannitol ^{13}C6	426.1 / 264.1	10
	426,1 / 336.14	10
bilobalide	299.1 / 271.1	10
	398.3 / 223.1	10
ginkgolide A	537.2 / 187.0	25
	537.2 / 391.2	10
ginkgolide B	625.3 / 479.2	10
	625.3 / 597.2	5
ginkgolide C	713.3 / 567.1	10
	713.3 / 595.1	10
ginkgolide J	478.3 / 264.2	10
	478.3 / 422.2	10

Based on these results we setup a quantitation method suitable for the monitoring of these analytes in a single data acquisition. **Figure 4** shows a representative MRM spectrum of ginkgotoxin, bilobalide, ginkgolide A, B, C, J and internal standard D-mannitol-^{13}C6 in MS quantitation mode. GC conditions were set to achieve baseline separation for all the four ginkgolides studied.

The data of calibration curves, i.e. retention times, ranges, R2 values, slopes are reported for each standard in Table 2. The results of the quantitative analysis are reported in **Table 3**. Based on our analysis we found that the commercial supplement contained 7,77% terpene lactones per tablet. The seed samples contained 50 ppm ginkgolide A and 14 ppm ginkgolide B, but ginkgolide C and J were below limit of quantitation (LOQ), methanol extracts of Ginkgo biloba cells from callus and cell suspension, MVs and NVs contained considerably less bilobalides and ginkgolides than the seed and supplement extracts. We

found that MVs sample contained 269 ppm, NVs 61 ppm, suspension culture (dry) 3 ppm, seed 266 ppm and tablet 37 ppm of ginkgotoxin.

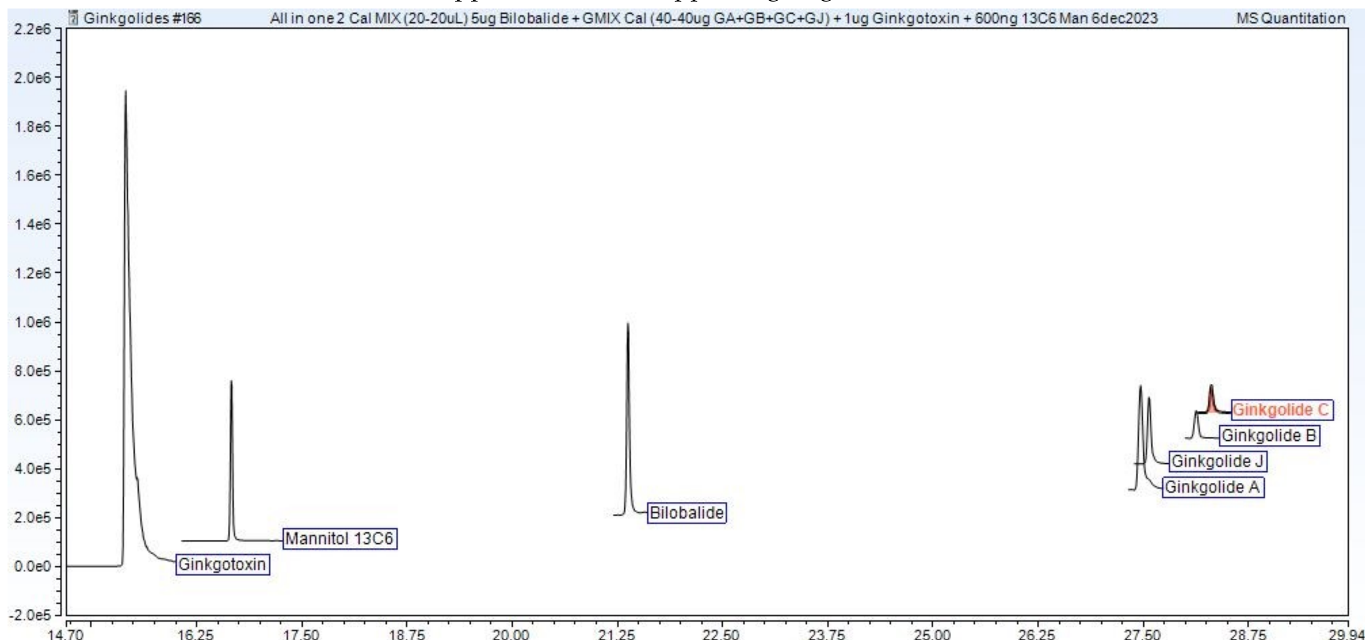

Figure 4. Representative MRM spectra of ginkgotoxin, bilobalide, ginkgolide A, B, C, J and internal standard the D-mannitol-^{13}C6 (MS quantitation).

Table 2. Calibration data (Retention Times (RT), Ranges, R^2, Slopes).

Compound	RT (min)	Calibration points	Range (µg)	R^2	Slope
ginkgotoxin	15.40	7	0.01-20	0.9944	13502.73
bilobalide	21.41	6	0.05-30	0.9917	343.02
ginkgolide A	27.53	5	0.50-50	0.9939	16.15
ginkgolide B	28.20	5	0.50-50	0.9965	14.44
ginkgolide C	28.35	5	0.50-50	0.9982	14.55
ginkgolide J	27.60	5	0.50-50	0.9804	14.76

Table 3. Results of the quantitative GC-MS/MS analysis of nanovesicles, microvesicles, cells from suspension culture wet and dry, callus wet and dry, seed and ginkgo supplement tablet – weight: mass used for the methanol extraction in milligrams (mg, average values); ginkgotoxin, bilobalide, ginkgolide A, B, C and J analytes expressed in part per millions (ppm) (average values). LOQ is for limit of quantitation.

Sample	Weight (mg)	Ginkgotoxin (ppm)	Bilobalide (ppm)	Ginkgolide A (ppm)	Ginkgolide B (ppm)	Ginkgolide C (ppm)	Ginkgolide J (ppm)
Nanovesicles (wet)	0.3	61	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Microvesicles (wet)	0.3	269	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Suspension culture (wet)	123.3-190.9	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Suspension culture (dry)	11.97	3	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Callus (wet)	127.8-182.9	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Callus (dry)	12.41	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Seed (dry)	49.20-52.04	266	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Tablet (dry)	274.05	37	11303	55525	4309	5008	1514

4. Conclusion

The ginkgolides are known to inhibit platelet aggregation, while the bilobalide protects against neuronal death caused by brain ischemia. On the other hand, ginkgotoxin is a neurotoxin structurally related to vitamin B6, naturally occurring in GB nuts and leaves. The untargeted full scan analysis of the different GB biomaterials could not identify any of the above, main GB-related secondary metabolites in the samples studied (**Appendix A**). These natural ingredients are present at very different quantities in the different GB derivatives and their simultaneous quantitation is neither trivial nor easy. The full scan analysis turned out to be useful in identifying primary metabolites (aminoacids, lipids, organic acids, aldehydes, ketones, alcohols, and sugars). Moreover, it identified two secondary metabolites, namely the flavonoid monomeric unit containing catechin (2R-trans) and epigallocatechin in both callus and suspension cell cultures. Cathenkins are known to be potent antioxidants that have already been identified in GB products. A recent study shows strong inhibition of β-amyloid peptide aggregation and destabilization of preformed fibrils by GB-derived cathekins and suggests that they represent viable approaches for the prevention and treatment of Alzheimer disease (Xie et al, 2014).
To achieve our goal towards the identification of the six GB secondary metabolites (**Figure 1**), we have setup a GC-MS/MS MRM method that can quantify these metabolites in a single run. To do that, we have improved the separation of 4 ginkgolides in the GC timescale by the careful setting of temperature ramping. Moreover, we have selected a pair of single reaction monitoring (SRM) precursor and product ions transitions for each analyte as well as optimized the collision energy for each transition (**Table 1**) and setup calibration curves for each analyte (**Table 2**). We found that the quantity of terpene lactones in grown ginkgo cells in vitro, MVs and NVs are lower than the quantitation limit (**Table 3**). In MVs we detected more ginkgotoxin than in NVs (**Table 3**). This could be explained by the lower purity grade of the MVs. In fact, MVs sample was obtained after a series of low velocity centrifugation steps from the GB seed homogenate, while the NVs were isolated after the ultracentrifugation step. Therefore, the higher amount of ginkgotoxin in MVs could be associated with its nonspecific association to the vesicles.
As a result of MRM analysis, the commercial supplement contained 7,77 % terpene lactones per tablet. The method could be useful for the quality control of similar products. The seed sample contained 50 ppm ginkgolide A and 14 ppm ginkgolide B, but ginkgolide C and J were below LOQ. Ginkgo biloba cells from callus and cell suspension, MVs and NVs contained considerably fewer bilobalide and ginkgolides than the seed and supplement extracts (**Table 3**). We measured that the quantity of ginkgotoxin were 269 ppm in the microvesicles samples, in NVs 61 ppm, suspension culture in dry condition 3 ppm, seed 266 ppm and tablet 37 ppm. This shows that the targeted MRM method can be useful for screening ginkgotoxin in different GB products.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki; blood was donated voluntarily by the authors of the study.

Conflicts of Interest: The authors declare no conflict of interest.

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Appendix A

1. Compounds identified in the Microvesicles sample in the full scan GC-MS analysis.

Compound	**compound class**
2,3-Butanediol	aldehyde, ketone and alcohol
Isoborneol	
D-Pinitol	
5-tridecaol	
2,6-Di-Tert-butylphenol	
cyclopropene-1-(2-Hydroxypropyl)-2-ol	
6-tridecanol	
Alanine	amino acid
Glycine	
Leucine	
13-Octadecenoic acid (E)	fatty acids
5,8,11-Eicosatrienoic acid, (Z)-	
9,12-Octadecadienoic acid (Z,Z)-	
Palmitic acid	
2-Butenedioic acid, (E)-	other organic acids
Butanedioic acid	
Citric acid	
Hydracrylic acid	
Lactic acid	
Oxalic acid	
1,2,2,3,4-Butanepentacarbonitrile	other compounds
4-Hydroxy-4-methylhex-5-enoic acid, tert.-butyl ester	
4-Pyridinol	
9-Octadecenoic acid, (2-phenyl-1,3-dioxolan-4-yl)methyl ester, cis-	
Octadecane, 6-methyl-	
Pyridinium, 1-(2-hydrazino-2-oxoethyl)-, chloride	
t-Butyldimethyl(2-styryl[13]dithian-2-yl	
carbamate	
Myo-Inositol	sugar
ß-D-Tagatopyranose	
α-D-Glucopyranoside, methyl 2-(acetylamino)-2-deoxy-3-OH-, cyclic methylboronate	
D-Mannitol	

2. Compounds identified in the nanovesicles sample in the full scan GC-MS analysis.

Compound	**Compound class**
2,6-DI-Tert-butylphenol	aldehyde, ketone and alcohol
Alanine	amino acid
Glycine	
Leucine	
L-Isoleucine	
L-Methionine	
L-Proline	
L-Threonine	
L-Valine	
Phenylalanine	
5,8,11-Eicosatrienoic acid, (Z)-	lipid
9,12-Octadecadienoic acid (Z,Z)-	
Palmitic acid	
Petroselinic acid	
Aconitic acid (E)	organic acid
Citric acid	
Hydracrylic acid	
Lactic acid	
1,2,2,3,4-Butanepentacarbonitrile	other
4-Hydroxy-4-methylhex-5-enoic acid, tert.-butyl ester	
4-Pyridinol	
5-Dimethy tridecane	
6-Dimethyl tetradecane	
Cephaloridine	
Decane	
D-Gala-l-ido-octonic amide	
Dodecane, 2,6,11-trimethyl-	
Isoborneol, pentamethyldisilanyl ether	
N-4,5-dihydrothiazol-2-amine	
Octadecane, 6-methyl-	
Pyridinium, 1-(2-hydrazino-2-oxoethyl)-, chloride	
Silanoltrimethyl-phosphate(3:1)	
t-Butyldimethyl(2-styryl[13]dithian-2-yl	
carbamate	
α-D-Glucopyranose	sugar
α-D-Glucopyranoside, methyl 2-(acetylamino)-2-deoxy-3-OH-, cyclic methylboronate	

3. Compounds identified in the ginkgobiloba seed sample in the full scan GC-MS analysis.

Compound	Compound class
(2R,3R,4R,5S)-Hexane-1,2,3,4,5,6-hexol	aldehyde, ketone and alcohol
2,3-Butanediol	
(S)-5-Methylhydantoin	
1,3,5-Pentanetriol	
2,6-DI-Tert-butylphenol	
Erythritol	
Ethanolamine	
Glycerol	
L-Arabinitol	
L-Threitol	
Propanetriol, 2-methyl-, tris-O-(trimethylsilyl)-	
trans-Coniferrylalcohol	
4-Aminobutanoic acid	amino acid
Alanine	
Asparagine	
DL-Pyroglutamic acid	
Glycine	
L-Aspartic acid	
Leucine	
L-Glutamic acid	
L-Isoleucine	
L-Methionine	
L-Proline	
L-Threonine	
L-Valine	
Phenylalanine	
Serine	
ß-Alanine	
11-Octadecenoic acid, (E)-	lipid
9(Z),11(E)-Conjugated linoleic acid trimethylester	
9,12-Octadecadienoic acid (Z,Z)-	
9-Octadecenoicacid (E)-	
Palmitic acid	
Stearic acid	
2-Butenedioic acid, (E)-	organic acid
Shikimic acid	
(E)-Ferulic acid	
2,3,4-Trihydroxybenzoic acid	
2-Amino-2-cyclopropylacetic acid	
2-Aminomalonic acid	
4-Hydroxybutanoic acid	
4-Trimethylsiloxy(trimethylsilyl)valerate	
Butanedioic acid	
Butanoic acid, 2,4-bis[(trimethylsilyl)oxy]-, trimethylsilyl ester	
D-Gluconic acid	
Glyceric acid	

Glycolic acid	
Lactic acid	
Malic acid	
Pentanedioic acid, 2-[(trimethylsilyl)oxy]-, bis(trimethylsilyl) ester	
Pyrrolidine-3-carboxylic acid	
Quininic acid	
Ribonic acid	
(3R,4S,5R)-3,4-Dihydroxy-5-((2S,3S)-1,2,3,4-tetrahydroxybutyl)dihydrofuran-2(3H)-one	
16-Heptadecyn-4-one, 1,2-dihydroxy-	
2,3-Dihydroxybutanedihydrazide	
2-Pyrrolidinone	
3-Vanil-1,2-bis(trimethylsilyloxy)propane	
5-Dimethyl(trimethylsilyl)silyloxytridecane	other
6-Dimethyl(trimethylsilyl)silyloxytetradecane	
Erythrono-1,4-lactone	
Methyl-3-(4-hydroxyphenyl)-2-[(4-hydroxyphenyl)methyl]-4-methoxy-5-oxofuran-2-carboxylate	
Pentasiloxane, dodecamethyl-	
Silanoltrimethyl-phosphate(3:1)	
Uridine	
1,5-Anhydroglucitol	
3-α-Mannobiose (isomer 1)	
D-(-)-Fructofuranose (isomer 1)	
D-(-)-Fructofuranose (isomer 2)	
D-(+)-Cellobiose (isomer 2)	
D-(+)-Talofuranose (isomer 2)	
D-(+)-Turanose	
D-Allofuranose	
D-Arabinopyranose (isomer 2)	
D-Glucitol	
D-Mannitol	
D-Pinitol	
Galactinol	
Galactitol	
Lactulose	sugar
Maltose	
Myo-Inositol	
ß-Arabinopyranose	
ß-D-(+)-Mannopyranose	
ß-D-(+)-Talopyranose	
ß-D-Galactopyranoside, methyl 2,3-bis-O-(trimethylsilyl)-, cyclic butylboronate	
ß-D-Galactopyranoside, methyl 2,3-bis-O-(trimethylsilyl)-, cyclic methylboronate	
ß-D-Glucopyranoside, 2-hydroxy-2-methyl-3-buten-1-yl	
ß-D-Glucopyranosiduronic acid, 3-(5-ethylhexahydro-2,4,6-trioxo-5-pyrimidinyl)-1,1-dime-	
ß-D-Tagatopyranose	
ß-Lyxopyranose	
Sucrose	
Sweroside	
Talose	
α-Chloralose (isomer 2)	

α-D-(+)-Talopyranose	
α-D-Glucopyranose	
α-D-Glucopyranoside, methyl 2-(acetylamino)-2-deoxy-3-OH cyclic methylboronate	
β-D-Lactose (isomer 2)	

4. Compounds identified in the cell suspension culture sample in the full scan GC-MS analysis.

Compound	Compound class
(S)-5-Methylhydantoin	aldehyde, ketone and alcohol
Ethanolamine	
Glycine	
4-Aminobutanamide	amino acid
Alanine	
Asparagine	
DL-Pyroglutamic acid	
Homoserine-4-imino-N,O-bis(trimethylsilyl)-trimethylsilylester	
L-5-Oxoproline	
L-Asparagine	
Leucine	
L-Glutamic acid	
L-Glutamine	
L-Isoleucine	
L-Proline	
L-Threonine	
L-Valine	
L-β-Homoglutamine	
Serine	
ß-Alanine	
9-Octadecenoicacid (E)-	lipid
Palmitic acid	
[(4,6-Diamino-2-pyrimidinyl)sulfanyl]acetic acid	organic acid
1-Aminocyclopropanecarboxylic acid	
2,3,4-Trihydroxybutyric acid	
2-Keto-L-gluconic acid	
4-Cyclohexene-1,2-dicarboxylicacid	
Butanedioic acid	
Glycericacid	
Lactic acid	
Malic acid	
Quininic acid	
Shikimic acid methyl ester	
3-Piperidineamin-N-(6-bromopyridin-2-yl)-1-trimethylsilyl-	other
3-Piperidinecarboxamide	
5-amino-3-isoxazolol	
D-(-)-Erythrose, tris(trimethylsilyl) ether, methyloxime (syn)	
Methyl-3-(4-hydroxyphenyl)-2-[(4-hydroxyphenyl)methyl]-4-methoxy-5-oxofuran-2-carboxylate	
Phosphoric acid, bis(trimethylsilyl)monomethyl ester	
Putrescine	

Silanoltrimethyl-phosphate(3:1)	
Stigmast-5-ene, 3β-(trimethylsiloxy)-, (24S)-	
Uracil	
Catechine(2R-trans)	secondary metabolite
Epigallocatechin	
3-α-Mannobiose (isomer 1)	sugar
a-D-Galactopyranose	
D-(-)-Fructofuranose (isomer 1)	
D-(-)-Fructofuranose (isomer 2)	
D-(+)-Turanose	
D-Altrose	
D-Fructose	
D-Glucopyranose	
D-Mannitol	
D-Psicopyranose (isomer 2)	
Galactinol	
Lactulose (isomer 1)	
Myo-Inositol	
ß-Arabinopyranose	
ß-D-Galactopyranoside, methyl 2,3-bis-OH-, cyclic methylboronate	
ß-D-Tagatopyranose	
ß-D-Xylopyranose	
ß-Lyxopyranose	
Sucrose	
Talose	
α-D-Galactopyranoside, methyl 2,6-bis-OH-, cyclic methylboronate	
α-D-Glucopyranose, 2,3,4,6-tetrakis-OH-phosphate	
α-D-Glucopyranoside, methyl 2-(acetylamino)-2-deoxy-3-OH-, cyclic methylboronate	

5. Compounds identified in the callus sample in the full scan GC-MS analysis.

Compound	Compound class
(S)-5-Methylhydantoin	aldehyde, ketone and alcohol
Ethanolamine	
1-Aminocyclopropanecarboxylic acid	amino acid
4-Aminobutanoic acid	
Alanine	
Asparagine	
DL-Pyroglutamic acid	
Glycine	
Homoserine	
L-Asparagine	
Leucine	
L-Glutamic acid	
L-Norvaline	
L-Proline	
L-Serine	
L-ß-Homoglutamine	
L-Threonine	
L-Valine	
Serine	
ß-Alanine	
ß-Glutamic acid	
Palmitic acid	lipid
2,3,4-Trihydroxybutyric acid	organic acid
D-Gluconic acid	
Glyceric acid	
Lactic acid	
Malic acid	
Quininic acid	
Shikimic acid	
(R)-1,1,1-Trimethyl-N-(1-oxybutan-2-yl)amine	other
3-Piperidineamin-N-(6-bromopyridin-2-yl)	
3-Piperidinecarboxamide	
5-amino-3-isoxazolol	
Methyl-3-(4-hydroxyphenyl)-2-[(4-hydroxyphenyl)methyl]-4-methoxy-5-oxofuran-2-carboxylate	
Putrescine	
Silanoltrimethyl-phosphate(3:1)	
Stigmast-5-ene, 3β-(trimethylsiloxy)-, (24S)-	
Catechine(2R-trans)	secondary metabolite
Epigallocatechin	
2-α-Mannobiose (isomer 1)	sugar
D-(-)-Fructofuranose (isomer 1)	
D-(-)-Fructofuranose (isomer 2)	
D-(+)-Turanose	
D-Altrose	
D-Fructose	
D-Mannitol	

D-Mannopyranose, phosphate	
D-Psicopyranose (isomer 2)	
Galactinol	
Myo-Inositol	
ß-Arabinopyranose	
ß-D-Galactopyranoside, methyl 2,3-bis-OH-, cyclic methylboronate	
ß-D-Tagatopyranose	
ß-D-Xylopyranose	
ß-Lyxopyranose	
Sucrose	
Talose	
α-D-Glucopyranoside, methyl 2-(acetylamino)-2-deoxy-3-OH-, cyclic methylboronate	
α-D-Mannopyranoside, methyl 2,3-bis-OH-, cyclic butylboronate	