Certificate of analysis

GINKGO BILOBA 24% PE

Batch N° : 257/09/A7

Ref : **146101**

	SPECIFICATIONS	METHODS	RESULTS
Organoleptic Quality :			
Appearance	Fluid powder	Visual: (CQ-MO-148)	conform
Color	Clear yellow	Visual: (CQ-MO-148)	conform
Flavor	Characteristic	Sensory: (CQ-MO-148)	conform
Solubility	Water soluble	Visual: (CQ-MO-148)	conform
Analytical Quality :			
Loss on drying :	< 3%	I.R.balance: (CQ-MO-018)	2,13%
Particle size :	100% through 60 mesh	Sieve: (CQ-MO-023)	100%
Flavone glycosides content :	> 24%	HPLC : (CQ-MO 223)	25,69%
Ginkgolides content :	> 6%	HPLC : (CQ-MO 282)	6,87%
Ginkgolic acid content :	< 5 ppm	HPLC : (CQ-MO 277)	< 5 ppm
Irradiation detection :	Not irradiated (PPSL<700)	PPSL	215
Microbiological Quality :			
Total Plate Count :	< 10000 cfu/g	Count: (CQ-MO-231)	Conform
Yeasts and moulds :	< 300 cfu/g	Count: (CQ-MO-244)	Conform
E.Coli :	negative/g	Count: (Ext Lab.)	Conform *
Salmonella :	negative/10g	Count: (Ext Lab.)	Conform *

Manufacturing date: September 14, 2007.
Expiration date: end of September 2009.

Date of issuance : September 24, 2007.

Julien SENEZ
Quality Control Manager

FRANCE : Site d'Agroparc - BP 1218 - 84911 AVIGNON Cedex 9 - Tél : 33 (0)4 90 23 96 89 - Fax : 33 (0)4 90 23 73 40
USA : 375 Huyler Street - SOUTH HACKENSACK, NJ 07606 - Phone : 1 201 440 5000 - Fax : 1 201 342 8000
SINGAPORE : 3 Pickering Street #03-24 - Nankin Row, China Square Central - SINGAPORE - 048660 - Phone: 65 6536 5168 - Fax: 65 6536 5178
E-mail : naturex@naturex.com - WebSite : www.naturex.com

	CQ-MO-223 Version : D Page 1/5 Date of first edition : September 16th, 2003

DOSAGE OF FLAVONOL GLYCOSIDES IN GINKGO BILOBA EXTRACTS BY HPLC

Revised edition	Title	Name	Date of revision	Visa
Written by :	Q.C. Technician	Mathieu TENON	07/22/05	
Checked by :	Q.C. Manager	Nicolas FEUILLERE	07/22/05	

1. FIELD OF APPLICATION

Determination of flavonol glucosides in Ginkgo biloba by High Performance Liquid Chromatography.

2. PRINCIPLE

This assay can be used to determine flavonol glycosides of Kaempferol, Quercetin, and Isorhamnetin in plant materials and dried extracts. The flavonol glycosides are first hydrolysed with hydrochloric acid to the aglycones, and the aglycones are assayed by HPLC. Correction factors are used to mathematically convert the aglycones to the corresponding flavonol glycosides.

3. EQUIPMENT AND REAGENTS

Equipment

- HPLC Agilent 1100, equipped with UV-Vis detector or equivalent
- Zorbax SB C18 HPLC column, 4.6 x 250 mm, or equivalent
- Ultrasonication bath
- Analytical balance
- 20 mL autosampler vial for headspace analysis
- 100, 50, and 20 mL volumetric flasks
- 5 and 10 mL volumetric pipets
- 0.45 µm PTFE syringe filter

Reagents:

- Methanol HPLC Grade
- Water HPLC Grade
- Phosphoric acid, 85% - ACS Grade
- Dimethylsulfoxide (DMSO)

Standards

- Quercetin from Extrasynthese - ref: 1135 S
- Isorhamnetin from Extrasynthese - ref: 1120 S
- Kaempferol from Extrasynthese - ref: 0054

4. CHROMATOGRAPHY

4.1 HPLC specification:

- Column : Zorbax SB C18 - 4,6 x 250 mm - 5 µm particle size or equivalent
- Column temperature : 35°C
- Flow Rate : 1.2 mL/ minute
- Detection : 270 nm
- Injection volume : 10 µl
- Mobile Phase: Methanol:0.5% phosphoric acid in water (50:50) isocratic gradient

Re-equilibrate at starting mobile phase conditions for at least 10 minutes after each run.

4.2 Nominal pressure:

The pressure allowed for this method is 200 Bar more or less 5 bar.

4.3 Retention Times:

Marker Compound	Retention time(min)	Relative retention
Quercetin	7.7	1.00
Kaempferol	12.9	1.67
Isorhamnetin	14.8	1.92

5. CALCULATIONS

5.1 Calibration curve for each standard flavonoides:

- Accurately weigh about: 4 mg of Isorhamnetin in a 20 ml volumetric flask
  10 mg of Kaempferol in a 20 ml volumetric flask
  6 mg of Quercetin in a 10 ml volumetric flask
- Dilute to volume with methanol HPLC grade (add 1 ml of DMSO for Isorhamnetin).
- Prepare the following dilutions: 1/2, 1/4
- Inject 10 µl of the stock standard solution and the linearity standard solution.
- Calculate the calibration curve by linear regression analysis for each component:

$$\text{Area} = a \times (c \times P) + b$$

Where: Area = Peak area of the component
c = concentration (g/L) of the component
a = Slope of the regression line
b = y-intercept of the regression line
P = purity of the standard given by the certificate of analysis from Extrasynthese

5.2 Amount of total Flavonol glycosides in sample:

5.2.1 Sample preparation

- Accurately weigh about 120 mg of the ginkgo dry extract into a 20 mL autosampler vial and add one drop of DMSO.
- Add 10 mL of 4% concentrated hydrochloric acid in methanol and cap.
- Heat at 60°C for 3 hours. The color of the solution is not a definitive indication of reaction completeness.
- Transfer the liquid in 50 mL volumetric flask and complete with methanol.
- Cool to room temperature and filter an aliquot through 0.45 µm PTFE syringe filter into an HPLC vial and cap.

5.2.2 Amount of Quercetin glycoside in the extract

$$\frac{A_Q - b_Q}{a_Q \times C\ (g/l)} \times \frac{756.70}{302.20} \times 100\%$$

Where: A_Q = Peak area of quercetin in the sample solution chromatogram
b_Q = y-intercept of quercetin calibration curve
a_Q = Slope of quercetin calibration curve
C = sample concentration (g/L)
756.70 = average formula weight of quercetin glycoside
302.2 = formula weight of quercetin dihydrate

5.2.3 Amount of Kaempferol glycoside in the extract

$$\frac{A_K - b_K}{a_K \times C\ (g/l)} \times \frac{740.7}{286.2} \times 100\%$$

Where: A_K = Peak area of kaempferol in the sample solution chromatogram
b_K = y-intercept of kaempferol calibration curve
a_K = Slope of kaempferol calibration curve
C = sample concentration (g/L)
740.7 = average formula weight of kaempferol glycoside
286.2 = formula weight of kaempferol

5.2.4 Amount of Isorhamnetin glycoside in the extract

$$\frac{A_I - b_I}{a_I \times C\ (g/l)} \times \frac{770.6}{316.2} \times 100\%$$

Where: A_I = Peak area of isorhamnetin in the sample solution chromatogram
b_I = y-intercept of isorhamnetin calibration curve
a_I = Slope of isorhamnetin calibration curve
C = sample concentration (g/L)
770.6 = average formula weight of isorhamnetin glycoside

316.2 = formula weight of isorhamnetin

5.2.5 Total Flavonol glycosides, in % w/w, in extract

$$Q + K + I$$

Where: Q = % Quercetin in the sample extract
K = % Kaempferol in the sample extract
I = % Isorhamnetin in the sample extract

6. SYSTEM SUITABILITY

6.1 Procedure

After equilibrating the HPLC system with the starting mobile phase conditions for at least 10 minutes, inject the diluent (methanol/hydrochloric acid 96:4) as a blank and verify that there are no interfering peaks and no carry-over. Make five injections of five different weight of the sample and determine the peak areas and retention time for each flavonoid in each chromatogram.

6.2 Component linearity in sample

Determine the correlation factor for each flavonoid in the sample solutions (peak area vs. component concentration in sample). The correlation factor of the curve for each of the compounds must be not less than 0.995. The %RSD of the sample curve must not be more than 2% (USP).

6.3 Tailing (USP)

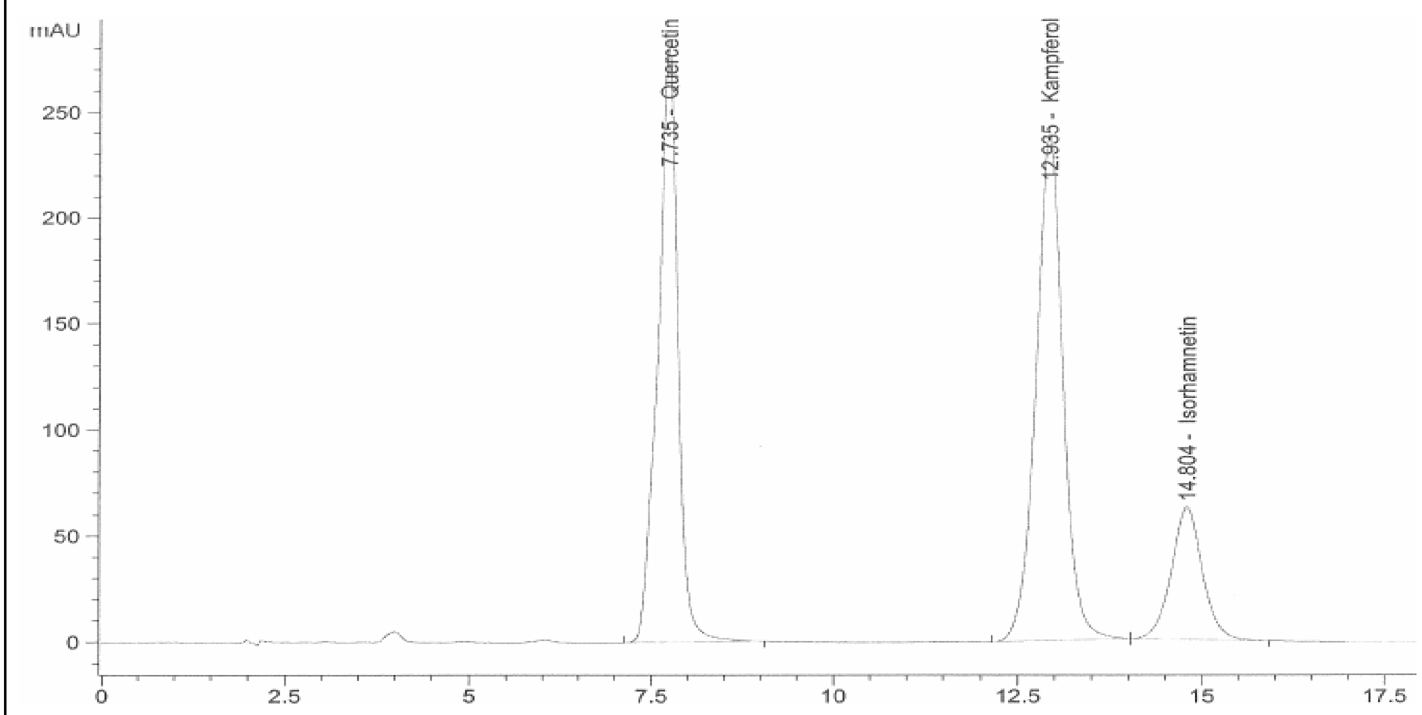
The tailing factor for all the components in the sample chromatogram must be between 0.90 and 1.30.

7. HISTORY OF REVISIONS

Date: *September 16th, 2004 Version B*; Change of eluent from water + 0.3%H3PO4 / methanol / acetonitrile (40:35:25) to methanol / 0.5% orthophosphoric acid (50:50) in accordance with INA METHOD 102.000.
May 19th 2005 Version C; Change in flavonol glycoside hydrolysis. No water is added.
July 22th, 2005 Version D; Add of "System suitability" for method validation.

8. ENCLOSED DOCUMENTS

8.1 Representative Standard chromatogram



8.2 Representative sample chromatogram

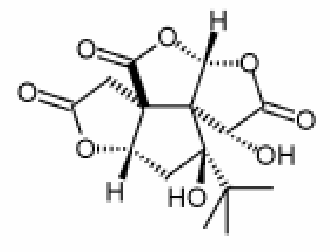
mAU
50
40
30
20
10
0
7.766 - Quercetin
12.976 - Kampferol
14.842 - sorhamnetin
0
2.5
5
7.5
10
12.5
15
17.5

DOSAGE OF GINKGOLIDES AND BILOBALIDE IN GINKGO BILOBA LEAVES EXTRACTS BY GAS CHROMATOGRAPHY.

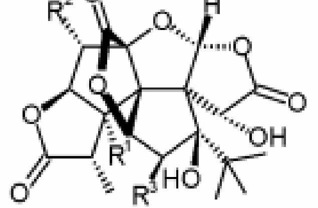
Edition	Title	Name	Date	Visa
Written by :	Q.C. Technician	Mathieu TENON	07/26/2007	
Checked by:	Q.C. Manager	SENEZ Julien	07/26/2007	

1. SCOPE AND FIELD OF APPLICATION

A method for the determination of the pharmacologically active terpene trilactones ginkgolide A,B,C and J and bilobalide (see figure 1) in Ginkgo biloba leaves extracts.



Bilobalide



Ginkgolide types

GK	R_1	R_2	R_3
A	OH	H	H
B	OH	OH	H
C	OH	OH	OH
J	OH	H	OH

Figure 1: chemical structure of terpene lactones from ginkgo biloba

2. PRINCIPLE

After extraction/concentration, the terpene lactones are determined by gas chromatography (after derivatization with BSTFA) on a ***TRB5*** column with FID detection. Lupeol a triterpene mono hydroxylated is used as an internal standard instead of squalane as we can see in most of publications.

Reference: *Selective dissolution and one step separation of terpene trilactones in ginkgo leaf extracts for GC-FID determination*, Q. Lang, H.Yak, C. Wai, Talanta 54 (2001) 673-680.

3. APPARATUS AND REAGENTS

Apparatus :

- GC Varian 3400 or equivalent with FID detector.
- CombiPal® autosampler
- Ultrasonic bath
- 20 mL tube for extraction
- 1;5 and 10 ml Pipets for eppendorf dispenser
- Syringe and filter 0,45 µm
- Analytical balance

Reagents :

- Water ***HPLC quality***
- Methanol ***HPLC quality***
- Sodium dihydrogen phosphate ***monobasic,monohydrate, ACS reagent***
- Tetrahydrofuran ***Chromasolv for HPLC***
- Ethyl acetate ***Normapur***
- Anhydrous sodium carbonate
- BSTFA+TMCS solution from Supelco
- Pyridine *puriss absolute*, over molecular sieve

Standard :

- Ginkgolide A from SIGMA ref : G4028
- Ginkgolide B from FLUKA ref: 05108
- Ginkgolide C from FLUKA ref: 18309
- Ginkgolide J is expressed as Ginkgolide B
- Bilobalide from SIGMA ref: B9031

4. PROCEDURE

4.1 Gas chromatography specification:

- Column : TRB-5 15 M x 0.32 mm – 0.25 μm or equivalent
- Column temperature : 200°C for 10 min then increase to 280°C at 4°C/min hold for 5 min finish at 320°C with 5 °C/min and hold for 5 min.
- Carrier gas: Nitrogen
- Injector temp: 260°C split (1/100) mode with liner type ref: 092028 from SGE company
- FID temp: 300°C, attenuation: 8 with range of 12
- Head pressure column: 8 Psi.
- Injector: 1μL

4.2 Stock solution:

-1- Internal stock standard solution (ISSS):

Accurately weigh about 10 mg of Lupeol internal standard into 10 mL volumetric flask and dilute to volume with THF/ethyl acetate (30:70 v:v). Keep refrigerate for 6 month.

-2- Stock standard solution (SSS):

Accurately weigh about 5 mg each ginkgolides and bilobalide into different 10 mL volumetric flask and dilute to volume with methanol. Keep at -20°C for 1 year.

4.3 Calibration curve for each standard:

- Working standard solutions:

Accurately weigh into six 20 mL glass autosampler vial the following volumes for each standard:

Labelled	*SSS* (µL)	ISSS(µL)
1	500	100
2	300	100
3	200	100
4	100	100
5	50	100
6	10	100

- Evaporate with nitrogen until dryness.
- Silylate under nitrogen with 200µL of BSTFA and 200µL of pyridine at 100°C for 2 hours.
- Inject 1 µL of these solutions, after cooling down into GC system.
- Record chromatogram of these six working standard solutions

Lupeol is used as an internal standard. Response factors were calculated for ginkgolide A,B and C and bilobalide by plotting for each of the ginkgo compounds the ratio of the area of the ginkgo peak to that of the internal standard peak against the ratio of the weight (concentration) of the ginkgo compound to that of the internal standard. The slope of each line gives the relative response factor (F_i).

$$F_{xi} = \Sigma(F_i) \ / 6$$

$$F_i = \frac{C_i}{A_b} \times \frac{A_i}{C_{is}}$$

where:
A_b: Area peak of compound calibrated
A_i: Area peak of lupeol
C_{is}: Weight of lupeol (µg)
C_i: Weight of compound (µg)
6 = number of injections

5. SAMPLES ANALYSIS

5.1 Sample preparation:

- Accurately weigh about 100 mg of the ginkgo leaf extract into 20 mL autosampler vial and dilute with 10 mL of 5% NaH_2PO_4 (w:v with water), Sonicate and mix vigorously in 50°C water bath for 10 min.
- Filter an aliquot on 0.45 µm and transfer 1 mL in 20 ml autosampler vial,
- Pipet 200 µL of ISSS and 2 mL of THF/ethyl acetate (30:70 v:v)
- Mix well with vortex and centrifuge at 2000 rpm for 2 minutes.
- Take 800 µL of the upper phase and evaporate to dryness under nitrogen

- Silylate under nitrogen with 200µL of BSTFA and 200µL of pyridine at 100°C for 2 hours.
- Inject 1 µL of this solution, after cooling down into GC system.

5.2 Total terpene lactones content:

Terpene lactone content (Q%) in samples is given by the formula:

$$Q(\%) = \sum \left[F_{xi} \times \frac{A_b}{A_i} \times \frac{C_{is}}{C_{spl}} \times d \right]$$

With:

A_b = Area peak of compound "I" quantified in sample
A_i = Area peak of Lupeol in sample
C_{is} = Amount of Lupeol (µg) in sample
C_{spl} = Weight of sample (mg)
F_{xi}= Response factor of the compound quantified determined in §4.3
d = 3 dilution factor

6. HISTORY OF REVISION

July 26th, 2007 Version B; Change in analytical method analysis from HPLC to GC.

7. SYSTEM SUITABILITY

7.1 Procedure

After equilibrating the GC system with the temperature conditions for at least 10 minutes, inject the blank and verify that there are no interfering peaks and no carry-over. Make six injections of six different weight of the sample and determine the Ginkgolide content in each chromatogram.
The RSD for this method is 2% (n =6) with a detection limit of 5 µg (injection 1 µL).

7.2 Tailing (USP)

The tailing factor (5%) for all the components in the sample chromatogram must be between 0.90 and 1.10.
Here for Bilobalide: 1.01, Ginkgolide A: 0.94, Ginkgolide B: 0.98, Ginkgolide C: 0.98, Lupeol: 0.99.

8. ENCLOSED DOCUMENTS

8.1 Representative Standard chromatogram

Bilobalide (9.332)
9.052
12.940
Ginkgolide A (24.939)
Ginkgolide B
Ginkgolide C (26.848)
Lupeol (29.276)
5
10
15
20
25
30

DOSAGE OF GINKGOLIC ACIDS IN GINKGO BILOBA EXTRACTS BY LIQUID CHROMATOGRAPHY.

Edition	Title	Name	Date	Visa
Written by :	Q.C. Technician	Mathieu TENON	06/12/2007	
Checked by:	Q.C. Manager	SENEZ Julien	06/12/2007	

1. SCOPE AND FIELD OF APPLICATION

Dosage of Ginkgolic acids (alkylphenol see figure 1) in ginkgo biloba extracts by High Performance Liquid Chromatography.

OH, COOH, R_2, R_1

1: $R_1 = C_{13}H_{27}$, $R_2 = H$
2: $R_1 = C_{15}H_{31}$, $R_2 = H$
3: $R_1 = C_{15}H_{29}$, $R_2 = H$
4: $R_1 = C_{17}H_{33}$, $R_2 = H$
5: $R_1 = C_{17}H_{31}$, $R_2 = H$
6: $R_1 = C_{15}H_{29}$, $R_2 = OH$
7: $R_1 = C_{17}H_{33}$, $R_2 = OH$

Figure 1: chemical structure of ginkgolic acids derivatives.

2. PRINCIPLE

HPLC quantification by external standard calibration against ginkgolic acid mixture and detection at 210 nm after separation on reverse phase C_{18} HPLC column.

References: USP method: Powdered ginkgo extract, *USP30NF 25* page 937.

3. EQUIPMENT AND REAGENTS

Equipment:
- HPLC Agilent 1100, equipped with UV-Vis detector or equivalent
- Hypersil ODS HPLC column, 2.1 x 100 mm, or equivalent
- Ultrasonication bath
- Analytical balance
- 10, 20, and 50 mL volumetric flasks
- 5 and 10 mL volumetric pipets
- 0.45 µm PTFE syringe filter

Reagents:
- Water HPLC grade
- Phosphoric acid
- Methanol HPLC grade
- Acetonitrile HPLC grade

Standards:

- Ginkgolic acid I (Ginkgolic acid C15:1 about 90% purity and Ginkgolic acid C13:0 mixture) from ChromaDex Reference: 07165

Ginkgolic acid C13:0 and Ginkgolic acid C17:1 are calculated against C15:1.

4. CHROMATOGRAPHY

4.1 HPLC specification:

- Column : Hypersil ODS - 100 x 2.1 mm - porosity : 3 µm; or equivalent
- Temperature : 35°C
- Flow : 1 ml/min
- UV detection : 210 nm, 8 nm
- Injection volume : 20 µl

- Eluent: linear gradient

Time (min)	% acetonitrile 0.1% H_3PO_4	%water 0.1% H_3PO_4
0	25	75
6	10	90
7	10	90
8	25	75
10	25	75

Re-equilibrate at starting mobile phase conditions for at least 10 minutes after each run.

4.2 Nominal pressure:

The pressure allowed for this method is 230 Bar more or less 5 bar.

4.3 Retention Times:

Marker Compound	Retention time(min)
Ginkgolic C13:0	3.3
Ginkgolic C15:1	3.6
Ginkgolic C17:1	5.3

5. CALCULATIONS

5.1 Calibration curve for each standard:

- Accurately weigh about: - 10 mg of Ginkgolic standard in a 20 ml volumetric flask

- Dilute to volume with methanol HPLC grade.

- Prepare the following dilutions: 1/2, 1/4.
- Inject 20 µl of the stock standard solution and the linearity standard solution.
- Calculate the calibration curve by linear regression analysis for each component:

$$Area = a \times (c \times P) + b$$

Where: Area = Peak area of Ginkgolic acid C15:1
c = concentration (g/L) of the component
a = Slope of the regression line
b = y-intercept of the regression line
P = purity of the standard given by the certificate of analysis from ChromaDex

5.2 Amount of Total Ginkgolic acid in sample:

5.2.1 Sample preparation:

- Dissolve 500 mg of Ginkgo biloba extract in 8 ml methanol. Sonicate and shake for 15 minutes.
- Dilute to 10 ml with water. Sonicate 5 minutes
- Filter on 0.45µm and inject 5 µl (or 20 µl for low content) of the sample.

5.2.2 Amount of total Ginkgolic acid in the extract:

$$\frac{A_G - b_G}{a_G \times C\ (g/l)} \times 100\%$$

Where: A_G = Peak area sum of Ginkgolic acid C13:0, C15:1 and C17:1 in the sample solution chromatogram
b_G = y-intercept of Ginkgolic acid C15:1 calibration curve
a_G = Slope of Ginkgolic acid C15:1 calibration curve
C = sample concentration (g/L)

6. SYSTEM SUITABILITY

6.1 Procedure:

After equilibrating the HPLC system with the starting mobile phase conditions for at least 10 minutes, inject the diluent (methanol) as a blank and verify that there are no interfering peaks and no carry-over. Make five injections of five different weight of the sample and determine the peak areas and retention time for each Ginkgolic acid in each chromatogram.

6.2 Component linearity in sample:

Determine the correlation factor for each of Ginkgolic acid in the sample solutions (peak area vs. component concentration in sample). The correlation factor of the curve for each of the compounds must be not less than 0.995. The %RSD of the sample curve must not be more than 2% (USP).

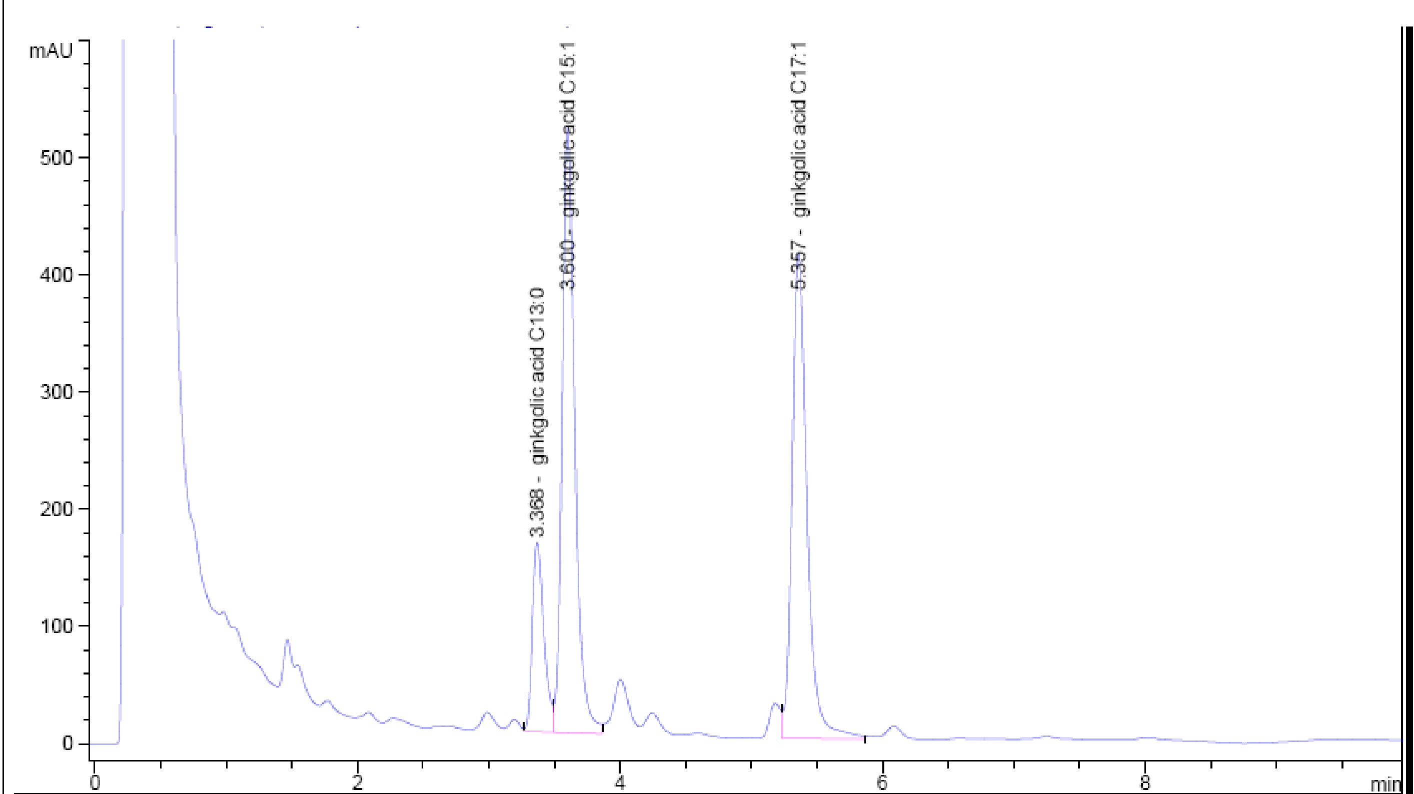
6.3 Tailing (USP):

The tailing factor for all the components in the sample chromatogram must be between 0.90 and 1.30.

7. HISTORY OF REVISION

August 13th, 2005 Version B; Added system suitability (§6.) and sample chromatogram figure.
June 12th, 2007 Version C; in accordance with USP.

8. ENCLOSED DOCUMENTS



Ginkgo sample chromatogram.