Direct Quantitation of Terpene Trilactones in *Ginkgo biloba* by Direct Analysis in Real Time (DART) and Flow Injection High Resolution Mass Spectrometry

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MSc. Thesis
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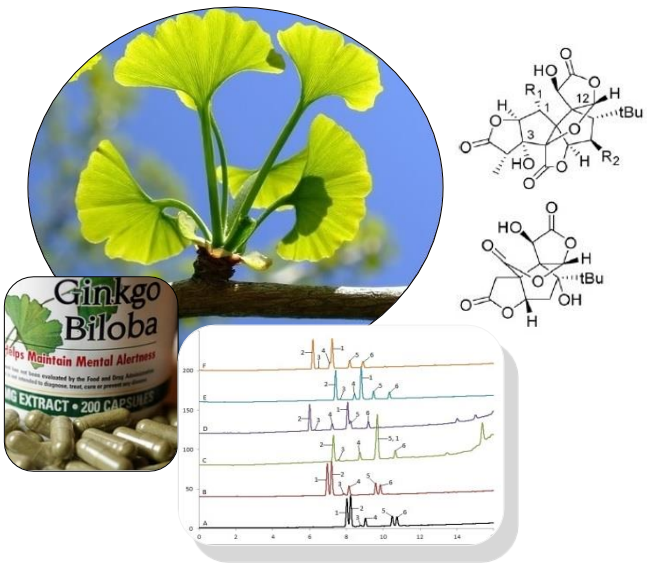

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Abstract

Direct analysis in real time-high resolution mass spectrometry (DART-HRMS) and flow injection-high resolution mass spectrometry (FI-HRMS) methods were evaluated for the determination of four major terpene trilactones in *Ginkgo biloba* extract (ginkgolides A, B, C and bilobalide). Picrotin was used as an internal standard for quantification. Initially, ionization modes were investigated to identify abundant ions that might be useful for the sensitive and selective detection of ginkgolides and bilobalide during HRMS measurement. Ginkgo extracts quantitation was performed by positive ionization in selected ion monitoring (SIM) mode that was carried out for ammonium adduct ions at m/z 426, 442, 458, 344 and 328 for ginkgolides A, B, C, bilobalide and picrotin respectively. As a benchmark for these methods, UHPLC-ELSD was applied firstly. Chromatographic separation of the analytes on a C18 column was performed using an isocratic elution of solvent methanol-tetrahydrofuran-water (8.8 : 5.76 : 85.44). Sample preparation and clean-up procedures were employed to enhance the chromatographic selectivity. DART-HRMS showed poor repeatability during standard measurement with RSDs range of 9.8 – 35.9%, while FI-HRMS resulted for good repeatability with RSDs range of 0.7 – 1.4%. Hence, FI-HRMS was used to analyse terpene trilactones contents in commercially *Ginkgo biloba* extracts. Sample clean-up procedures were skipped by taking the advantages of the selectivity of MS detection. Analyte recoveries during reached more than 95%, the intra-assay RSDs were < 8%, it is able to detect 2.5 µg/mL terpenoid compounds and sample assay was carried out within 1.5 min measuring time. However, the developed method was lack in specificity due to the absence of separation feature and has an uncertain accuracy and sensitivity that is promising to be explored for further study. The accuracy was low as the deviation between the developed method and the benchmark reached 26.2%. Therefore, this FI-HRMS assay is quite promising to be explored to perform a rapid and reliable analysis of ginkgolides A, B, C and bilobalide in *Ginkgo biloba* extracts.

Keywords: Ginkgo biloba, terpene trilactones, bilobalide, ginkgolides, UHPLC-ELSD, direct analysis in real time-high resolution mass spectrometry, DART-HRMS, flow injection-high resolution mass spectrometry, FI-HRMS.

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Chapter 1. Introduction

1.1 *Ginkgo biloba* and its bioactive compounds

Ginkgo biloba is the oldest living tree which belongs to a member of Ginkgoaceae family that still exists, whereas all other species within its division have become extinct (Major, 1967). It was grown throughout China and Korea, was introduced into Japan, then into Europe and to North America. *Ginkgo biloba* has been widely used for herbal medicine prescription by ancient Chinese ancestors from the use of ginkgo fruits (Strømgaard and Nakanishi, 2004). In 1965, the German company introduced the preparations of *Ginkgo biloba* leaf to the Western world under the trade name Tebonin. Later, a standardised *Ginkgo biloba* extract was developed in Germany which was called EGb761 and has brand names such as Tanakan, Rokan and Tebonin forte (McKenna et al., 2001) which contains 24% flavonoid glycosides, 6% terpene lactones, and less than 5 ppm ginkgolic acid (Smith and Luo, 2003, Jacobs and Browner, 2000). This content had become a benchmark for qualitative standard for most of commercial standardized *Ginkgo biloba* extracts (Mauri et al., 1999).

Ginkgo biloba has been used in traditional herbal medicine prescriptions by using its seeds or leaves. Ginkgo seeds were used in China to treat pulmonary disorders such as asthma, cough and enuresis; alcohol abuse and bladder inflammation while the leaves have been mainly used to treat heart and lung dysfunctions and skin infections (Smith and Luo, 2003). In Germany, around 1960s, leaf extracts EGb761 was used in the treatment of blood circulation disorders both peripherally and centrally (Van Beek et al., 1998). The consumption of ginkgo extracts helps in the treatment of Alzheimer's disease and improving memory (Kanowski et al., 1996, Maurer et al., 1997). A comprehensive study showed that Ginkgo may slowdown cognitive decline during dementia. This result has a conjunction with the function of ginkgo as an antioxidant and its role in alleviating some chronic diseases (Fillit et al., 2002).

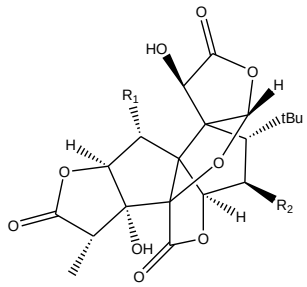
Nowadays, the consumption of herbal medicine products increases with the raise of public awareness in the use of natural products to prevent or cure some diseases (Neldner, 2000). As an alternative tool to prevent diseases, the production of herbal medicine has grown faster. In this case, *Ginkgo biloba* has become the most sold and most studied medicinal plants which ranks among the 10 most popular botanical dietary supplements (Mar and Bent, 1999). With retail sales of US $ 150 million, gingko leaf extract which was sold in 1998, has been ranked 7th on the best-selling herbal product list (Izzo and Ernst, 2001).

As previously mentioned, the most abundant compound in *Ginkgo biloba* is flavonoid followed by terpene trilactones (TTLs). TTLs are able to penetrate the blood brain barrier due to the presence of the lipophilic group and becomes partially responsible for the effect in the cerebra spinal sites. In addition, ginkgolides can selectively inhibit the platelet activating factor (PAF) whether bilobalide has neuroprotective properties (van Beek et al., 1998, Bruno et al., 1993). Thus, TTLs becomes a concern in every study about the quality control of *Ginkgo biloba* extracts (Strømgaard and Nakanishi, 2004) considering the presence of unique chemical compounds namely bilobalide and ginkgolide A, B, C and J (van Beek and Montoro, 2009).

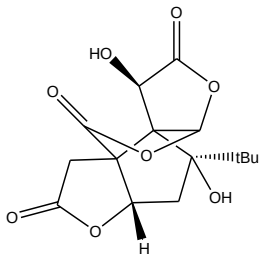
Isolation and identification process of TTLs content in *Ginkgo biloba* were carried out in

different periods. Ginkgolides, which were identified as C_{20} compounds, were isolated and characterized in 1960 from the root bark of the ginkgo tree (Maruyama et al., 1967). Later, bilobalide which is the most abundant TTL in ginkgo extracts (Strømgaard and Nakanishi, 2004) with around 2.9 % of the total standardized Ginkgo leaf extract (Smith and Luo, 2003) was identified in the leaves (Nakanishi et al., 1971). Furthermore, ginkgolide J as a minor TTL was isolated from the leaves in 1987 (Strømgaard and Nakanishi, 2004).

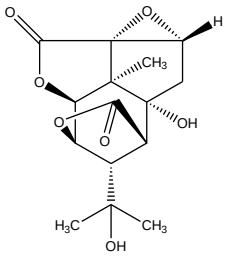
Ginkgolides belong to diterpene compounds consisting of six five-membered rings: a spiro[4.4]-nonane carbocyclic ring, three lactones and a tetrahydrofuran ring. Besides that, they contain a unique tert-butyl group. The variation is only in their hydroxyl groups both in the number and positions. Meanwhile, bilobalide as a part of sesquiterpene group also contained the characteristic tert-butyl group, as well as a secondary and a tertiary hydroxyl group (Strømgaard and Nakanishi, 2004). Compared with ginkgolides, it also contained three lactones and a tert-butyl group, but only one carbocycle (Nakanishi et al., 1971). The structures of these terpenes are given in Figure 1. Another sesquiterpene compound, picrotin, was used as the internal standard since it has a similar molecular structure as ginkgolides and bilobalide.



G-A: R1 = R2 = H, MW: 408.4
G-B: R1 = OH, R2 = H, MW: 424.4
G-C: R1 = R2 = OH, MW: 440.4
G-J : R1 = H, R2 = OH, MW:424.4



bilobalide, MW: 326.3



picrotin, MW: 310.3

Figure 1. Molecular structures of ginkgolides, bilobalide and picrotin.

1.2 Methods used to assay bioactive compounds of *Ginkgo biloba*

Chromatographic methods such as HPLC and GC were employed to identify and quantify TTLs in *Ginkgo biloba* extracts. These methods should fulfil the requirements as selective since they can separate the five individual TTLs and quantify their contents afterwards (van Beek and Montoro, 2009). GC with flame ionization detection (FID) has been used to analyse TTLs in which derivatisation should be employed before the measurement (Lolla et al., 1998). The most widely used method is HPLC since it can separate the five TTLs with good resolution within less than 15 min and detected with adequate sensitivity. In addition, no derivatisation of analytes is necessary during sample preparation and also a high availability of this equipment in QC labs. (Van Nederkassel et al., 2005). Further application of UHPLC can be employed for TTLs analysis in which it will reduce time used in the analysis measurement (van Beek and Montoro, 2009). The most available column used is C18 while the solvent commonly used is the combination between methanol and THF, and only rarely used of acetonitrile, propanol, isopropanol or isobutanol modifiers (van Beek and Montoro, 2009).

The use of UV as a detector in HPLC measurement is not recommended since TTLs have very low ε-values at their maximum absorbance, around 219 nm (Van Beek, 2005). Since TTLs do not contain conjugated carbon, they lack common chromophores and therefore UV detection is not suitable (van Beek, 2002). This raises a problem during the detection in which peaks of TTLs are easily overshadowed by impurities which absorb UV strongly, commonly come from flavonoid compounds in the *Ginkgo biloba* extracts (van Beek and Montoro, 2009).

Refractive index (RI) has also been employed as an alternative detection method (Van Beek et al., 1991). However, for routine analysis of TTLs, ELSD detector is the most popular device. It has better baseline stability over RI, compatible with THF and gradients, small solvent peak and higher sensitivity (van Beek and Montoro, 2009). ELSD was also successfully applied to quantify TTLs in *Ginkgo biloba* extracts. This detector has a non-linear behaviour in the response, thus it needs at least five calibration points to perform a linear logarithmic calibration curve (Tang et al., 2003). Furthermore, like UV and RI detection, it lacks selectivity. Thus, sample clean-up becomes a necessity and a proper HPLC parameter settings are necessary to eliminate impurities during the sample analysis (Sun et al., 2005).

LC/MS/MS analysis of Ginkgo dietary supplement is quite promising since it measurers without the need of a sample clean-up as proposed by Tang et al., 2003 in developing HPLC-ELSD method. LC/MS combines very high selectivity with adequate sensitivity (van Beek and Montoro, 2009). A highly sensitive and rapid method for the analysis of complex mixture such as plant extracts, can be performed using an electrospray ionization (ESI) interface (Camponovo et al., 1995). Drawbacks of MS for routine TTLs quality control are related to its performance for quantitative work in which not every type of mass spectrometer can be applied equally. Meanwhile, it has a complex operational features and costly for initial purchase and maintenance (van Beek and Montoro, 2009). Another method that can be employed for identification of TTLs is 1H NMR-based method. However, this method shows a lack of sensitivity and is slow compared to LC/MS assays (Sun et al., 2005).

Recently, DART-HRMS has become an alternative method to analyse bioactive compounds in plant extracts. DART-HRMS is an analytical method that can be facilitated in ambient environment analysis, allowing for the rapid and direct analysis of almost any type of samples without cumbersome preparation (Kim and Jang, 2009). DART-HRMS was applicable to distinguish between Chinese star anise and toxic Japanese star anise without any prior sample pretreatment (Shen et al., 2012). Electrospray ionization (ESI) became a favourable ionization mode for polar analytes determination since it has a smooth ionization process.

1.3 Experimental background and objective

HPLC-ELSD becomes the most widely used method for routine analysis of terpene trilactones in *Ginkgo biloba*. Meanwhile, it needs more concern in terms of the use of high amounts of chemicals as the solvent and time consuming. Moreover, it has a lack of selectivity when it is used to measure a sample with a complex mixture of natural compounds in plant extracts. Thus, this analytical method requires complex sample preparation and sample clean-up to eliminate the impurities from plant extracts and keep the longevity of the column.

A former research on DART-HRMS application in rapid control of Chinese star anise fruits produced nice results in which a toxic compound of Japanese star anise as the contaminant can be determined obviously (Shen et al., 2012). Furthermore, considering the difference of TTLs molecular weight, it is assumed that direct MS determination and quantification of TTLs in Ginkgo biloba extract are able to be carried out. Hence, in present study, DART-HRMS and FI-HRMS methods were developed and optimised for direct MS identification and quantification of TTLs. Further investigation was done related to sample pretreatment whether involve or ignore the need of sample clean-up prior to analysis. The absence of sample preparation was not sure in advance and taken into account during the experiment.

To see the performance of these methods, UHPLC-ELSD was established first as a benchmark in the sample quantitation step. The use of UHPLC instead of HPLC has an excellence in which it has less time and solvent consumption. However, MS based method requires an expensive instrument and the use of UHPLC also more expensive than HPLC.

Chapter 2. Materials and Methods

2.1 Materials

Terpene trilactones (TTLs) contained bilobalide (BB) and ginkgolide A, B and C (G-A, G-B and G-C) were provided by Organic Chemistry Lab., Wageningen University.
Picrotin 97% as the internal standard (IS) was purchased from ABCR GmbH & Co.KG, Karlsruhe, Germany.
Enriched *Ginkgo biloba* dry extract was provided by Organic Chemistry Lab., Wageningen University:

Name : Ginkgo Terp (2012 B29).
Exp. Date : 09-01-2015
Content avg. : G-A 27.85%, G-B 11.92%, G-C 5.50% and BB 47.92% (based on the NMR assay)

Ginkgo biloba dry extract (GBE) samples were provided by Organic Chemistry Lab., Wageningen University:

Sample	Identity
Sample 4	Extr. *Ginkgo biloba* e fol. sicc., Finzelberg Extrakte, Andernach
Sample 5	Extr. Ginkgo bil. e fol. sicc., Finzelberg Extrakte, Andernach
Sample 7	*Ginkgo biloba* extract 24%, Mfg. by Japan Greenwave Ltd., Chemco Industries Inc.

Analytical grade methanol (MeOH) was purchased from Sigma-Aldrich Laborchemikalien GmbH, USA.
Tetrahydrofuran unstabilized (THF) and ethyl acetate (EtOAc) were purchased from Biosolve BV, the Netherlands.
High purity deionized water (18 MΩ) was prepared by using Milli-Q water purification system (Waters, Milford, MA, France).
Sodium dihydrogenphosphate monohydrate p.a. (NaH_2PO_4, MW: 137.99 g/mol) was purchased from Acros Organics New Jersey, USA.
Sodium chloride p.a. (NaCl, MW: 58.44 g/mol) was purchased from Merck KGaA, Darmstadt, Germany.
Ammonium chloride (NH_4Cl, MW: 53.491 g/mol) was purchased from Merck KGaA, Darmstadt, Germany.

Syringes used to transfer solutions during the standard dilution:
- Syringe 0.50 mL, Vici precision sampling, Baton Rouge, Lousiana, USA: for transferring 275–500 µL solution.
- Syringe 0.25 mL, Vici precision sampling, Baton Rouge, Lousiana, USA: for transferring 50–250 µL solution.
- Syringe 0.025 mL, Hamilton Co., Reno, Nevada, USA: for transferring 5–25 µL solution.

2.2 Preparation of terpene trilactones (TTLs) standard and internal standard (IS) solutions

G-A, G-B, G-C and BB stock solutions (2000 µg/mL) were prepared in MeOH. 20.00 mg of each compound were weighed into a 10.0 mL volumetric flask, dissolved and diluted to volume. Picrotin stock solution (4000 µg/mL) was prepared in MeOH by weighing

40.00 mg of picrotin into a 10.0 mL volumetric flask. These solutions were stored in the fridge at temperature of –18 ºC.

Single working standard solutions were prepared for UHPLC-ELSD, DART-HRMS and FI-HRMS optimisations. From TTLs standard stock solutions, 125.0 µL of the solution was transferred into 10.0 mL volumetric flask separately. From picrotin stock solution, 62.5 µL was transferred into 10.0 mL volumetric flask. MeOH was added until the mark. The final concentration of the single working standard solutions was 25 µg/mL.

A mixture of 25 µg/mL TTLs and picrotin was prepared by transferring 125.0 µL of TTLs stock solutions and 62.5 µL of picrotin into a 10.0 mL volumetric flask and diluted with MeOH until the mark. This solution was used to optimise the parameters setting of UHPLC-ELSD, DART-HRMS and FI-HRMS.

Mixed working standard solutions for UHPLC-ELSD measurement were prepared in different concentration levels. Stock solution of ginkgolides and bilobalide were diluted to get final concentration in the range of 25–400 µg/mL for G-B and G-C, 50–550 µg/mL for G-A and 250–850 µg/mL for BB. Calibration solutions were prepared as follow:

- From G-B and G-C standard stock solutions, 12.5, µL were transferred and mixed into 1.0 mL volumetric flask (25 µg/mL calibration solution).
- 25, 50 and 87.5 µL of G-A, G-B and G-C standard stock solutions were transferred and mixed into 1.0 mL volumetric flasks (20, 100 and 175 µg/mL calibration solutions).
- 125, 162.5 and 200 µL of G-A, G-B, G-C and BB standard stock solutions were transferred and mixed into 1.0 mL volumetric flasks (250, 325 and 400 µg/mL calibration solutions).
- 275 µL of G-A and BB standard stock solutions were transferred and mixed into 1.0 mL volumetric flask (550 µg/mL calibration solution).
- 350 and 425 µL of BB standards stock solution was transferred into 1.0 mL volumetric flasks (700 and 850 µg/mL calibration solutions).

Furthermore, 50 µL of picrotin stock solution was added into each flask precisely. To each flask, MeOH was added until the mark. The resulting concentrations of the TTLs standard solutions were 25, 50, 100, 175, 250, 325, 400, 550, 700 and 850 µg/mL respectively. The final concentration of the IS in each aliquot was 200 µg/mL.

Mixed working standard solutions for DART-HRMS and FI-HRMS were prepared by diluting stock solutions into intermediate working solutions. 2.5 mL of G-A, G-B and G-C standard stock solutions were transferred into separate 10.0 mL volumetric flask. These solutions were diluted with MeOH to obtain a 500 µg/mL intermediate working solutions. From BB standard stock solution, 5.0 mL of the solution was transferred into a 10.0 mL volumetric flask and diluted with MeOH to obtain a 1000 µg/mL intermediate working solution. From IS standard stock solution, 1.00 mL of the solution was transferred into a 10.0 mL volumetric flask and diluted with MeOH to obtain a 400 µg/mL intermediate working solution. These intermediate working solutions were diluted to get a final concentration in the range of 2.5–70 µg/mL for G-A, G-B and G-C; and 5–140 µg/mL for BB by transferring 5, 10, 20, 50, 80, 110 and 140 µL of the solutions and mixed into 1.00 mL volumetric flasks. To each of these flasks, 50.0 µL of 400 µg/mL picrotin was added quantitatively. In order to enhance the ammonium adduct fragment ion species, 100 uL of 40 mM ammonium chloride was added into the solutions. Finally, MeOH was added to reach a total volume of 1.0 mL. The resulting concentrations of the G-A, G-B and G-C

calibration solutions were 2.5, 5, 10, 25, 40, 55 and 70 µg/mL, while the concentrations of BB were 5, 10, 20, 50, 80, 110 and 140 µg/mL respectively. All of these aliquots contain 20 µg/mL of IS and 4 mM of NH_4Cl.

40 mM of NH_4Cl was prepared by weighing 214.0 mg of NH_4Cl into 100.0 mL volumetric flask and diluted with MeOH until the mark.

2.3 UHPLC-ELSD solvent optimisation

UHPLC-ELSD was used as the benchmark of DART-HRMS and FI-HRMS measurements. Hence, a proper solvent used to separate TTLs with good resolution becomes a necessity. DryLab software was used to optimise the solvent composition by applying three different gradient systems. These systems have two different time gradients (fast and slow gradients: 6 and 18 min) and two different column temperatures (25 and 50 ºC). To optimise the chromatographic condition, analytes resolutions were recorded in MeOH, THF and water solvents. The summary of the solvent optimisation system is figured in Table 1. The retention time of the analytes were related to the combination between different solvents, gradient times and column temperatures.

Table 1. Chromatographic systems optimised to obtain raw chromatograms as the input data for DryLab software application.

System 1	System 2	System 3
A: 100% H_2O	A: 100% H_2O	A: 100% H_2O
B: 100% MeOH	B: 80% THF	B: MeOH-THF-H_2O (5 : 4 : 1)
Grad. : 6 and 18 min	Grad. : 6 and 18 min	Grad. : 6 and 18 min
Temp. : 25 and 50 ºC	Temp. : 25 and 50 ºC	Temp. : 25 and 50 ºC

System 3 represented the middle position between system 1 and system 2 which contains either MeOH or THF in their half portion. The gradient elutions were programmed with an increase from 5% of B at the initial time until 95% of B at the end of gradient time with a flow rate of 0.3 mL/min. An equilibration time of 4 min was allowed before each injection. Within these fast and slow gradient systems, the columns were thermostated at 25 and 50 ºC. In total, 12 profiles of chromatograms were obtained, which provided data about retention time of each analytes in each gradient system.

The data of retention times obtained from the 12 chromatogram profiles were plotted into DryLab software. The determination of peak retention times must be done properly by injecting single compound solutions as a confirmation.

2.4 Terpene trilactones analysis by UHPLC-ELSD

The ginkgolide chromatograms were performed using a Reverse Phase Ultra High Pressure Liquid Chromatography (RP-UHPLC) system (Agilent Technologies 1290 Infinity) equipped with a pump, an autosampler and an evaporative light scattering detection (ELSD) detector. Before analysis, all samples were filtered using 0.45 µm filter paper to remove solid particles. 1.0 µL of sample was injected into a Zorbax Eclipse Plus shield C18 column (2.1 x 100 mm, 1.8 µm particle size; Agilent, USA). The temperature of the column was controlled at 52 ºC during the analysis. The ELSD detector of SEDEX 90 (Sedere LT-ELSD, France) was set to measure at temperature 40 ºC with gain 10 in sensitivity and a pressure of 3.9 bar. A mixture of MeOH-THF-water (8.8 : 5.76 : 85.44) was used as a mobile phase at a flow rate of 0.4 mL/min in isocratic system with an 11

min running time. To remove some impurities from the plant constituents of *Ginkgo biloba* extract samples, 5 min column flushing using 60% MeOH was enforced. Then, 4 min equilibration time for column conditioning using the solvent was performed before the next injection. Quantification of ginkgolides and bilobalide were performed by calculating the peak area ratio between TTLs and the added IS; the calibration curve was made from a series of terpene trilactones; 25–400 µg/mL for G-B and G-C, 50–550 µg/mL for G-A and 250–850 µg/mL for BB; with IS 200 µg/mL.

2.4.1 UHPLC-ELSD repeatability test

A system suitability test was performed to see the performance of the device to assay sample whether qualitatively or quantitatively with a reliable result. The RSDs value of area ratio between TTLs and internal standard from 10 times injection of a 25 µg/mL standard mixture solution was evaluated as a parameter. Furthermore, RSDs value of retention time also takes into account to ensure that the peaks are positioned in a reproducible retention time.

2.4.2 UHPLC-ELSD linearity test

As the system produced reproducible data, the evaluation was continued with a linearity test to ensure that the ELSD detector gives a response proportionally related to the concentration of solutions introduced into the system. The linearity test was performed using different concentration points within the range of 25–400 µg/mL for G-B and G-C, 50–550 µg/mL for G-A and 250–850 µg/mL for BB; with IS 200 µg/mL. The calibration curve was constructed by plotting the log of area ratio of TTLs to IS versus the log of concentration of each analyte.

2.5 Terpene trilactones analysis by DART-HRMS

The direct mass spectrometry analysis was performed on a direct analysis in real time-high resolution mass spectrometer (DART-HRMS). DART ion source was set in both positive and negative mode ionisation at temperature 400 ºC. For the positive mode, DART ion source was set at capillary temperature 250 ºC, capillary voltage +25V, tube lens voltage +95V and skimmer voltage +28V. DART settings in negative mode: capillary temperature 240 ºC, capillary voltage –50V, tube lens voltage –135V and skimmer voltage –40V. Helium was used as sheath gas and nitrogen was used as auxiliary gas at a flow rate of 20 mL/min and 10 mL/min respectively. Mass spectrometer used was an Exactive high-resolution mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). The resolution was set at "high" over an m/z range of 200–800 Da. 1.0 µL of sample was applied onto a sample compartment containing metal mesh and the measurement was performed by continuous speed at 0.4 mm/min.

Data acquisition and processing were done with Xcallibur 2.1.0 (Thermo Scientific). Quantification method was performed by the calculation of peak area ratio between TTLs and the added IS in positive ionisation modes. The area ratio was performed by dividing the total peak area of $[M+NH_4]^+$ and $[M+H]^+$ between ginkgolides and IS.

2.5.1 DART-HRMS repeatability test

Repeatability test was performed by measuring a 25 µg/mL standard mixture solution containing internal standard 10 times and the RSDs values of area ratio between TTLs and internal standard were evaluated.

2.6 Terpene trilactones analysis by FI-HRMS

The mass spectrometry analysis was performed on an Exactive high-resolution mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). The system was equipped with an electrospray ionisation (ESI) probe coupled to a liquid chromatography (LC; Finnigan Surveyor, Thermo Scientific, San Jose, CA, USA) with a 1.0 µL valve system and was tuned with terpene trilactones. Helium was used as sheath gas and nitrogen was used as auxiliary gas with a flow rate of 20 L/min and 10 L/min respectively. MeOH as a mobile phase was pumped directly from the LC at 250 µL/min. The resolution was set at "high" over an m/z range of 200–800 Da. The spray voltages were 3.5 and 3.2 kV for positive and negative ion analysis respectively. Ion source was set at capillary temperature of 275 ºC for both positive and negative mode. For the positive mode, capillary voltage was set at +25V, tube lens voltage +190V and skimmer voltage +16V. Meanwhile, in negative mode it was set at capillary voltage –32.5V, tube lens voltage –135V and skimmer voltage –40V.

Data acquisition and processing were done with Xcallibur 2.1.0 (Thermo Scientific). Quantification method was performed by the calculation of peak area ratio between TTLs and the added IS in positive ionisation mode. The area ratio was performed by dividing the peak area of $[M+NH_4]^+$ between ginkgolides and IS; the calibration curve was made of a series of terpene trilactones; 2.5–70 µg/mL for G-A, G-B and G-C; and 10–140 µg/mL for BB with IS 20 µg/mL and 4 mM NH_4Cl.

2.6.1 FI-HRMS repeatability test

Repeatability test was performed by measuring a 25 µg/mL standard mixture solution contains internal standard 8 times and the RSDs values of the area ratio between TTLs and internal standard were evaluated.

2.6.2 FI-HRMS linearity test

The linearity test was performed using different concentration points within the range of 2.5–70 µg/mL for G-A, G-B and G-C; and 10–140 µg/mL for BB. The calibration curves were constructed by plotting the area ratio of TTLs to IS versus the concentration of each analyte.

2.7 Relative recovery test by UHPLC-ELSD and FI-HRMS assay

Relative recovery test was observed to see the recovery performance of liquid-liquid extraction, by comparing the obtained amount of analytes after liquid-liquid extraction to those with direct methanol extraction.

Firstly, a mixture of compounds containing 240 µg/mL of G-A, G-B and G-C; 560 µg/mL of BB and 320 µg/mL of picrotin was prepared (E). Of this (E) solution, 1.00 mL was

transferred into a 2.0 mL volumetric flask (in triplicate) and diluted with MeOH until the mark (F). This (F) solution was filtered through a syringe filter and ready for UHPLC-ELSD assay. For FI-HRMS assay, 1.00 mL of (F) solution was transferred into a 10.0 mL volumetric flask (in triplicate), added with 1.00 mL 40 mM NH_4Cl and diluted with MeOH until the mark.

For liquid-liquid extraction, 5.0 mL of (E) solution was transferred into a capped centrifuge tube (in triplicate) and evaporated by blowing nitrogen over the solution. 5.00 mL of 5% NaH_2PO_4 pH 4.27 was added and sonicated at 50 ºC for 1 hour. After it was cooled to a room temperature, 1.0 g of NaCl and 5.00 mL of EtOAc–THF (4+1, v/v) was added. The solution was shaken to dissolve the salt. Then it was shaken at 2000 rpm for 1 min to extract the analytes from the aqueous phase.

After both phases separated, 1.00 mL of organic phase was transferred into a 2.0 mL volumetric flask. A gentle stream of nitrogen was introduced to evaporate the organic phase into dry in a laboratory fume hood. Methanol was added until the mark to dissolve the dried extract (G). Finally, the filtrate of (G) was filtered through a syringe filter and ready for UHPLC-ELSD quantification. From (G) solution, 1.00 mL was transferred into a 10.0 mL volumetric flask, added with 1.00 mL 40 mM NH_4Cl and diluted with MeOH until the mark. The solution was ready for FI-HRMS measurement.

2.8 Sample preparation for UHPLC-ELSD and FI-HRMS measurements: internal standard method

2.8.1 Methanol extraction

An accurately weighed 15.0 mg of enriched ginkgolides powder was diluted with 5.00 mL MeOH in 10.0 mL volumetric flask. 0.500 mL of picrotin stock solution (4000 µg/mL) was added into the solution, homogenised with 2000 rpm vortex for 1 min and diluted with MeOH until the mark (A). After the solid particle settled, the supernatant of (A) solution was filtered through a syringe filter and ready for UHPLC-ELSD quantification. The prepared sample contained 200 µg/mL of internal standard picrotin as a correction. From (A) solution, 1.00 mL was transferred into a 10.0 mL volumetric flask, added with 1.00 mL of 40 mM NH_4Cl solution and diluted with MeOH until the mark. The final solution was ready for FI-HRMS assay (contained 20 µg/mL picrotin). For sample quantitation purpose, sample was weighed thrice (n = 3) and the average value was considered representative for the ginkgolides and bilobalide content.

An accurately weighed 250.0 mg of commercially *Ginkgo biloba* extract was treated under the same procedures as mentioned above. However, the final solution only being assayed by FI-HRMS due to the necessity of sample clean-up procedure before injecting into UHPLC-ELSD instrument.

2.8.2 Liquid-liquid extraction of terpene trilactones

An accurately weighed 15.0 mg of enriched ginkgolides powder or 250.0 mg commercially ginkgo extract powder was mixed with 5 mL of 5% NaH_2PO_4 solution pH 4.27 in a 10.0 mL volumetric flask. 0.500 mL of picrotin stock solution was added to achieve 200 µg/mL concentrations at the end of the extraction process. This solution was sonicated at 50 ºC for 1 hour. After the temperature cooled down into a room temperature,

5% NaH_2PO_4 was added to reach the volume. After the solid particle settled, the supernatant was used for liquid-liquid extraction. For sample quantitation purpose, sample was weighed thrice.

5.00 mL of sample prepared with 5% NaH_2PO_4 was transferred into a capped centrifuge tube, then 1.0 g NaCl and 5.00 mL EtOAc–THF (4+1, v/v) were added and shaken to dissolve the salt. The capped tube was shaken at 2000 rpm for 1 min to extract the terpene trilactones from the aqueous solution into the organic phase.

After both phases separated, 2.00 mL of organic phase was transferred into a vial. A gentle stream of nitrogen was introduced to evaporate the organic phase to dry in a laboratory fume hood. 2.00 mL methanol was added to dissolve the dried extract (B). Finally, the filtrate of (B) was filtered through a syringe filter and ready for UHPLC-ELSD quantification. From (B) solution, 1.00 mL was transferred into a 10.0 mL volumetric flask, added with 1.00 mL 40 mM NH_4Cl and diluted with MeOH until the mark. The solution was ready for FI-HRMS measurement. Each solution was measured in triplicate and the average value (n = 3) was considered representative for the ginkgolides and bilobalide content.

2.8.3 Calibration curve ranges

Calibration curves prepared for UHPLC-ELSD assay were within the range of 25–400 µg/mL for G-B and G-C, 50–550 µg/mL for G-A and 250–850 µg/mL for BB. All of these calibration solutions contained 200 µg/mL picrotin as an internal standard.

Calibration curves prepared for FI-HRMS assay were within the range of 2.5–70 µg/mL for G-A, G-B and G-C, whereas BB calibration curve range was prepared within 10–140 µg/mL. All of these calibration solutions contained 20 µg/mL picrotin as an internal standard.

2.9 Sample preparation for UHPLC-ELSD and FI-HRMS measurements: standard addition method

2.9.1 Methanol extraction

For MeOH extraction, an accurately weighed 18.0 mg of enriched ginkgolides powder was diluted with 2.00 mL MeOH in a 5.0 mL volumetric flask and homogenised with 2000 rpm vortex for 1 min. MeOH was added until the mark (A).

Un-spiked solution was treated by transferring 0.500 mL of (A) solution into a 2.0 mL volumetric flask (in triplicate). 0.500 mL of picrotin solution containing 400 µg/mL was added into the solution. Then, MeOH was added until the mark (B). This (B) solution was filtered through a syringe filter and ready for UHPLC-ELSD quantification. From (B) solution, 1.00 mL was transferred into a 5.0 mL volumetric flask, added with 0.500 mL of 40 mM NH_4Cl solution and diluted with MeOH until the mark. The final solution was ready for FI-HRMS assay.

For spiked solution, 0.500 mL of (A) solution was transferred into the other 2.0 mL volumetric flask (in triplicate), added with the same amount of picrotin and 0.500 mL of

standard mixture containing 600 µg/mL of BB and 200 µg/mL of G-A, G-B and G-C. This solution was treated under the same procedure as the un-spiked sample.

A commercially Japan *Ginkgo biloba* extract was weighed 500.0 mg and enforced with the same procedure as done as for an enriched ginkgolides powder, both for un-spiked and spiked solutions. For these extract, the assay was done using FI-HRMS only instead of UHPLC-ELSD since this sample needs to be cleaned up using liquid-liquid extraction.

2.9.2 Liquid-liquid extraction of terpene trilactones

For sample extraction, an accurately weighed 625.0 mg of Japan *Ginkgo biloba* extract was diluted with 15.00 mL 5% NaH_2PO_4 solution pH 4.27 in a 25.0 mL volumetric flask. This solution was sonicated at 50 ºC for 1 hour. After the temperature cooled down into a room temperature, 5% NaH_2PO_4 was added to reach the volume (C).

Un-spiked solution was done by transferring 3.00 mL of (C) solution into a capped centrifuge tube (in triplicate) and 0.500 mL of picrotin solution containing 400 µg/mL was added into the solution. Then 0.6 mg NaCl and 3.00 mL EtOAc–THF (4+1, v/v) were added and shaken to dissolved the salt. The capped tube was shaken at 2000 rpm for 1 min to extract the terpene trilactones from the aqueous solution into the organic phase.

After both phases separated, 2.00 mL of organic phase was transferred into a vial. A gentle stream of nitrogen was introduced to evaporate the organic phase into dry in a laboratory fume hood. 2.00 mL methanol was added to dissolve the dried extract (D). Finally, the filtrate of (D) was filtered through a syringe filter and ready for UHPLC-ELSD quantification. From (D) solution, 1.00 mL was transferred into a 5.0 mL volumetric flask, added with 0.500 mL 40 mM NH_4Cl and diluted with MeOH until the mark. The solution was ready for FI-HRMS measurement.

For spiked solution, 0.500 mL of (C) solution was transferred into the other 2.00 mL volumetric flask (in triplicate), added with the same amount of picrotin and 0.500 mL of standard mixture containing 600 µg/mL of BB and 200 µg/mL of G-A, G-B and G-C. This solution was treated under the same procedure as the un-spiked sample.

2.10 Instrument assays

2.10.1 UHPLC-ELSD measurement

Solutions prepared for chromatography assay were measured in triplicate by injecting 1.0 µL sample solution into the instrument. The average of these data was taken into account as the representative data for quantitation work.

2.10.2 DART-HRMS measurement

Solutions prepared for DART-HRMS assay was measured in 10 replications by depositing 1.0 µL of the solution onto the surface of mesh sample compartment. The average of these results was used as a representative data for quantitation work.

2.10.3 FI-HRMS measurement

Sample prepared for mass spectrometric assay was measured in 3 replications for FI-HRMS measurement by injecting 1.0 µL sample solution into an injector connected to a liquid chromatograph coupled with electrospray ionisation probe. The average of these results was taken into account as a representative data for quantitation work.

2.11 Calculations

2.11.1 Repeatability test

The value of RSDs of peak area ratios of TTLs to IS was evaluated to see the instrument repeatability performance by UHPLC-ELSD, DART-HRMS and FI-HRMS.

2.11.2 Linearity test

Linear regression procedure was used to analyse the linearity of each standard curve by examining the value of R^2 obtained.

2.11.3 Precision

The precision was expressed using RSDs from three assays within the measurement, correlates with the repeatability of the method.

2.11.4 Relative recovery

The relative recovery was determined by evaluating the obtained analytes after liquid-liquid extraction compared to the methanol extract. The mean recovery was calculated on three assays for pure standards and expressed as the percent difference between the averaged measurements and the nominal values.

2.11.5 t-test analysis of UHPLC-ELSD and FI-HRMS in terpene trilactones quantitation performance

To see the performance of FI-HRMS as a quantitation method to assay terpenoid content in *Ginkgo biloba* extract, an evaluation test was done by comparing the result assay with those from UHPLC-ELSD as a benchmark. The output data from the comparison is *t*-value which then compared with *t*-table, to see whether their result was differing significantly or not.

The formulas as follows:

$$S = \sqrt{\frac{(n_1 - 1).s_1^{\,2} + (n_2 - 1).s_2^{\,2}}{(n_1 + n_2 - 2)}}$$

$$t = \frac{(x_1 - x_2)}{S\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

S = pooled standard deviation
$n_1 = n_2 = 3$
s_1 = standard deviation method 1
s_2 = standard deviation method 2
x_1 = mean value method 1
x_2 = mean value method 2
t = t-value
t-table = 2.78 (d.f = 4, P = 0.05%)
d.f = degree of freedom = 3 + 3 – 2 = 4
(from triplicate data)

2.11.6 FI-HRMS performance in terpene trilactone assay compared to UHPLC-ELSD

The current study aims to see the performance of FI-HRMS method in quantifying ginkgolides and bilobalide content in *Ginkgo biloba* extracts with UHPLC-ELSD, the routine analysis method, as a benchmark. Therefore, the parameters used in the evaluation should be established as a representative to give the remark of its performance. The evaluation was based on the following criteria:

Table 2. The criteria used to assess FI-HRMS method performance.

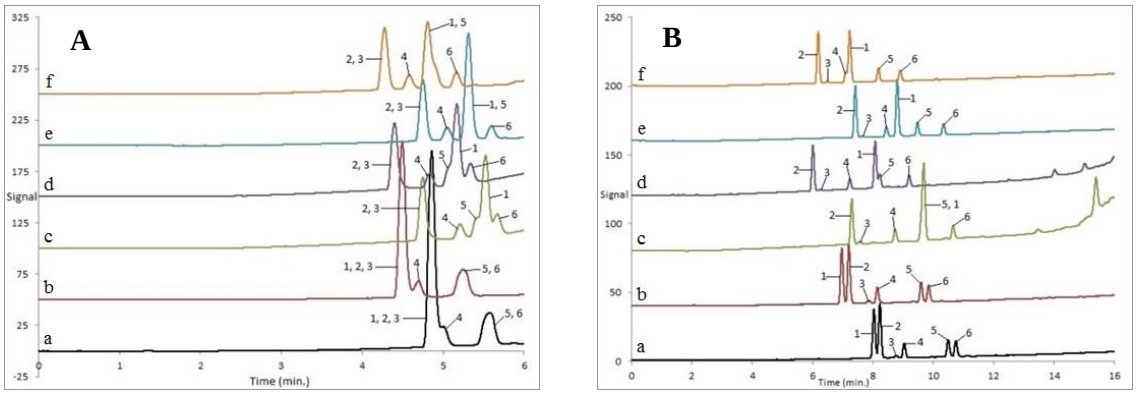
Criterion	Definition
Accuracy	The deviation of analytical result compared to the NMR data.
Time	The amount of time needed to prepare the sample solution and measure the sample from the injection up to data integration.
Cost	Chemicals used during sample preparation prior to analysis and chemicals used during the measurement with the related method. In addition, spare parts of the instrument also taken into account.
Reproducibility	Ability to provide a reproducible data, represented by the low RSDs value (< 10%) for supporting sample quantitation work.
Specificity	Ability to distinguish between terpene trilactones and their impurities from plant constituents.
Sensitivity	The smallest quantity of terpene trilactones that can be detected.

Chapter 3. Results

3.1 Selection of UHPLC-ELSD conditions

During the development of HRMS method, UHPLC-ELSD was employed as benchmark for sample quantitation work. Thus, the condition of this method should be established first, in particular for the solvent composition which can separate TTLs compounds and the internal standard used as well. The use of solvent with a composition of MeOH–THF–water (21 : 10.5 : 68.5) separated TTLs compounds, but picrotin as the internal standard is coeluted with G-J. Thus, a proper solvent composition should be optimised firstly.

As a tool for solvent optimisation, DryLab was employed. Computer simulation program is valuable for the efficiency of method development and maximize information about method specificity. It required data about the retention times of the compounds within different solvent systems. The profile of chromatograms can be seen in Figure 2, while the detail of their retention time is presented in Appendix 1.



a: Solvent H2O - 100% MeOH, temperature 25 ºC
b: Solvent H2O - 100% MeOH, temperature 50 ºC
c: Solvent H2O - 80% THF, temperature 25 ºC
d: Solvent H2O - 80% THF, temperature 50 ºC
e: Solvent H2O - (50% MeOH-40% THF-10% H2O), temperature 25 ºC
f: Solvent H2O - (50% MeOH-40% THF-10% H2O), temperature 50 ºC

1. Bilobalide
2. Picrotin
3. G-J
4. G-C
5. G-A
6. G-B

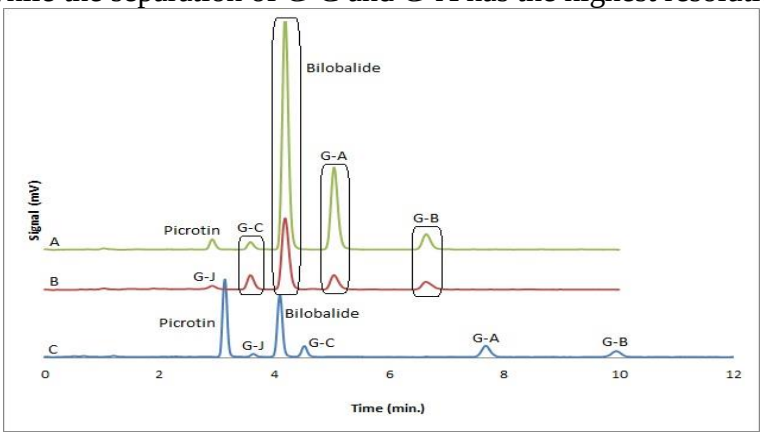
Figure 2. Chromatogram profiles of analytes after running with 6 min gradient (A) and 18 min gradient (B) at 0.3 mL/min flow rate.

In this work, 12 initial input experiments were performed with three different solvents, two different column temperatures and two different time gradients. The data were imported into DryLab software and an isocratic separation was predicted. The software simulation showed the optimum separation within 12 min run time with the following conditions:

- flow rate = 0.4 mL/min
- column temperature = 52 ºC
- solvent mixture = 100% methanol–80% THF (55 : 45)
- final solvent composition = water–solvent mixture (84 : 16)

After the application of DryLab software based on the retention times obtained from chromatograms above, the final solvent composition was established as MeOH–THF–water (8.8 : 5.76 : 85.44) with the column temperature of 52 ºC at 0.4 mL/min flow rate. The final chromatograms can be seen in Figure 3. From this picture, it can be seen that

picrotin and G-J that was coeluted when using the former solvent (Figure 4A and 4B) finally can be separated by employing the new solvent composition (Figure 4C) with retention time of 3.15, 3.65, 4.10, 4.53, 7.70 and 9.97 for picrotin, G-J, BB, G-C, G-A and G-B respectively. The actual retention times for each component in the mixture shows resolution values ≥ 1.5 with the lowest resolution was observed by the separation of BB and G-C, 1.6, while the separation of G-C and G-A has the highest resolution value, 8.5.



A & B: Solvent MeOH–THF–water (21 : 10.5 : 68.5)
C: Solvent MeOH–THF–water (8.8 : 5.76 : 85.44)

Figure 3. Chromatogram profiles of analytes with the former solvent composition (A and B) and using the final solvent composition (C).

3.2 UHPLC-ELSD repeatability test

The repeatability of the peak area ratios of TTLs to IS from ten times injected standard mixture solution was about 1.8–3.0% for intra-day analysis, as depicted in Table 3. Furthermore, the RSD values of peak retention times are recorded to see the peak position stability. For all of the peaks, they have RSDs value 0.1%.

Table 3. UHPLC-ELSD repeatability test of terpene trilactones and picrotin.

Analyte	Area ratio ± SD	RSD (%)	RT ± SD	RSD (%)
G-A	0.64 ± 0.02	3.0	7.43 ± 0.01	0.1
G-B	0.51 ± 0.01	1.8	9.58 ± 0.01	0.1
G-C	0.75 ± 0.01	2.0	4.35 ± 0.00	0.1
BB	0.86 ± 0.02	2.1	3.91 ± 0.00	0.1
Picrotin	-	-	3.02 ± 0.00	0.1

*n = 10; SD = standard deviation
RSD = relative standard deviation

As the ELSD detector resulted in a reproducible data, the system is ready to be used for analysing calibration curves and quantifying the sample of *Ginkgo biloba* extract.

3.3 UHPLC-ELSD linearity test

In order to check the performance of ELSD response, the linearity test was delivered using several levels of reference compound mixtures. Detector response for ELSD is given by $y = ax^b$, where y is the peak area ratio of TTLs to IS and x is the sample concentration. Therefore, calibration curves were plotted from logarithm of concentration versus logarithm of peak area ratio (Tang et al., 2003). Linear ranges and correlation coefficients for each terpene trilactones are given in Table 4 (see Appendix 2 for graph details).

Table 4. Linear ranges and correlation coefficients (r^2) of terpene trilactones by UHPLC-ELSD assay.

Analyte	Linear range (µg/mL)	Calibration curve	Correlation coefficient (r^2)
G-A	50 – 550	y = 1.36956x – 3.16779	0.99804
G-B	25 – 400	y = 1.22102x – 2.89895	0.99544
G-C	25 – 400	y = 1.32206x – 3.06193	0.99176
BB	250 – 850	y = 1.56036x – 3.63072	0.99937

3.4 Sample clean-up of *Ginkgo biloba* extract prior to UHPLC-ELSD analysis

UHPLC-ELSD method as a routine quantitative control of terpene trilactones can be used as a "quality indicator" to evaluate commercial *Ginkgo biloba* extracts. However, GBE has complex composition since it is produced from Ginkgo leaves or barks that have some herbal constituents. Therefore, sample clean-up was needed to remove some natural impurities that might interfere with the elution of TTLs analytes.

During the extraction, 5% NaH_2PO4 was used as the aqueous phase to dissolve the sample, based on the previous research by Lang et al., 2004 which validated sample clean-up procedures to extract TTLs in *Ginkgo biloba* samples using 5% KH_2PO4. This buffer solution at 50–60 ºC with ultrasonication gave very clean chromatograms (Lang et al., 2004)

Peak identifications were made by comparing the retention times of the samples with those of the reference compounds. The difference between chromatogram sample before and after sample clean-up can be seen in Figure 4. From picture 4A it can be seen that G-B has lower peak response after sample clean-up was employed. It supposed to be the removal of some impurities around this peak after the liquid-liquid extraction was performed.

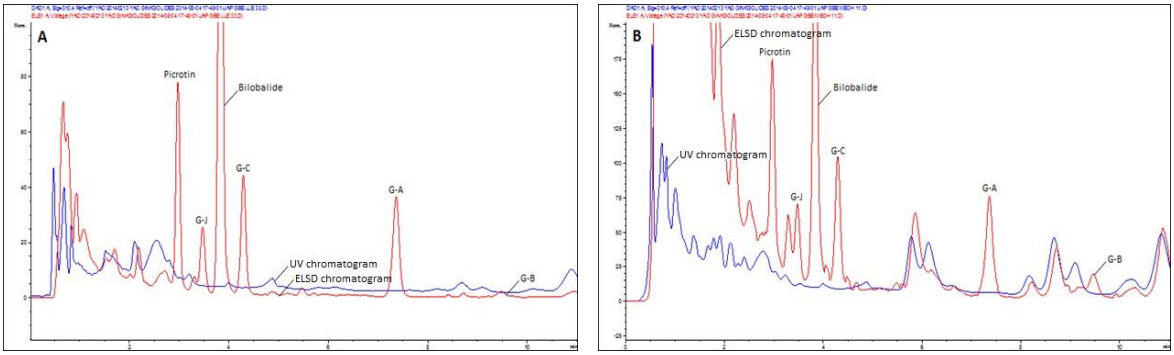


Figure 4. Chromatograms obtained by UHPLC-ELSD for Japan *Ginkgo biloba* extract with (A) and without (B) sample clean-up.

3.5 DART-HRMS analysis of terpene trilactones

DART-HRMS was applied for the first time for *Ginkgo biloba* terpene analysis. Sharp resolution of the compounds can be produced from both positive and negative ionisation modes with high resolution scan type.

For negative ionisation mode, more than one specific fragmentation occurred. The fragmentation produced a specific mass related to the loss of tert-butyl compound with 56 Da mass (van Beek, 2005). In negative ion mode, there were no adducts and the typical

mass ion obtained by SIM was the $[M-H]^-$ ion. The typical ions detected for each terpenoid compound can be seen in Table 5, whereas the mass spectrum is presented in Figure 5.

Table 5. Typical ions detected for terpene trilactones and picrotin using DART-HRMS in negative ionisation mode.

	G-A	G-B	G-C	BB	IS
Molecular mass (Da)	408	424	440	326	310
$[M-H]^-$	407	423	439	325	309
$[M\text{-tert-butyl}]^-$	351	367	383	-	-

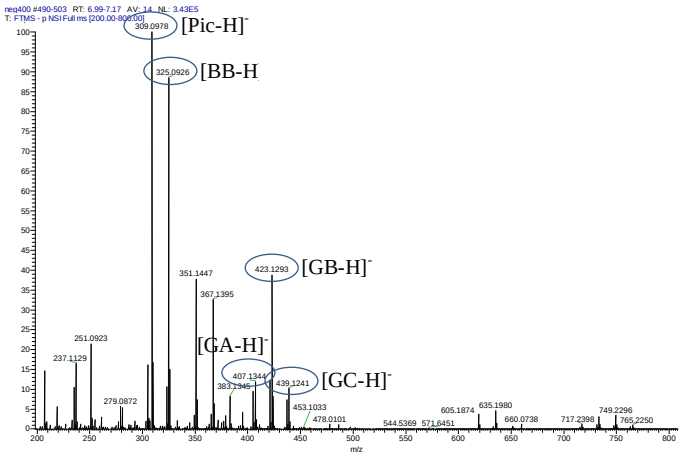


Figure 5. Typical mass spectrum of terpene trilactones and picrotin of 5 µg/mL pure ginkgolides mixture obtained by DART-HRMS in negative ionisation mode.

For positive ionisation mode, the typical mass spectrum of terpene trilactones and picrotin as the internal standard obtained by SIM (selected ion monitor) were ammonium adduct species $[M+NH_4]^+$ and protonated ion $[M+H]^+$. The typical ions detected for each terpenoid compound are summarized in Table 5. In order to enhance the abundance of ammonium adduct ion, the addition of 10 mM NH_4Cl has been evaluated. However, the profile of TTLs spectrum was remained the same as without the addition of this modifier with the presence of the protonated ion, as can be seen in Figure 6.

Table 6. Typical ions detected for terpene trilactones and picrotin using DART-HRMS in positive ionisation mode.

	G-A	G-B	G-C	BB	IS
Molecular mass (Da)	408	424	440	326	310
$[M+NH_4]^+$	426	442	458	344	328
$[M+H]^+$	409	425	441	327	311

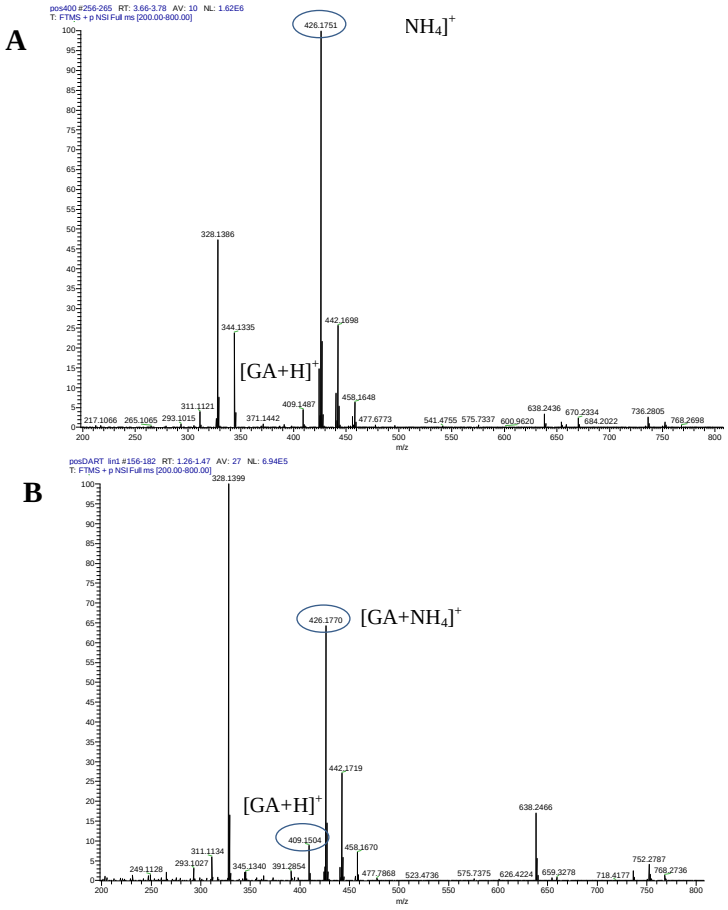


Figure 6. Typical mass spectrum of terpene trilactones and picrotin of 5 µg/mL pure ginkgolides mixture obtained by DART-HRMS in positive ionisation mode without the addition of NH_4Cl (A) and after the addition of NH_4Cl (B).

The ion abundance after the addition of NH_4Cl is lower compared to the solution without the presence of this modifier, $6.94.10^5$ instead of $1.62.10^6$. Hence, solution used for quantitation work was a mixture of TTLs and picrotin without the addition of NH_4Cl.

3.6 DART-HRMS repeatability test

Based on the optimisation results, positive ionisation mode becomes an option to assay terpene trilactones content in *Ginkgo biloba* extract. Prior to sample quantitation analysis, repeatability test should be carried out to ascertain that DART-HRMS instrument could deliver a reproducible data.

Total ion chromatograms obtained have been integrated into selective ion chromatograms. It corresponded to the mass of terpenoid compounds both in $[M+NH_4]^+$ and $[M+H]^+$ ions. For instance, G-B that has a mass of 424 Da has m/z 442 and 425 quantitation ions, whereas picrotin as the internal standard that has a mass of 310 Da has m/z 328 and 311 quantitation ions (Figure 7). Further, the areas of these selective ion chromatograms have been used in RSDs calculation. G-B area ratio was obtained by dividing the total area of $[GB+NH_4]^+$ and $[GB+H]^+$ ions by the total area of $[Pic+NH_4]^+$ and $[Pic+H]^+$ ions. From the repetition measurement, it can be seen that the chromatograms have a significant difference profile over time. The repeatability test result can be seen in Table 7.

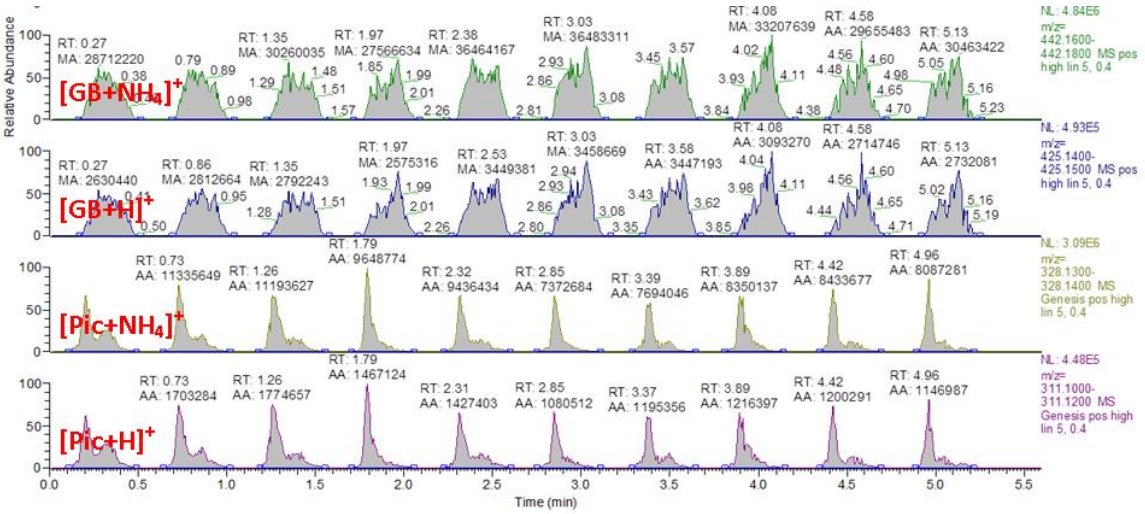


Figure 7. Extracted ion chromatograms of ginkgolide B and picrotin 25 µg/mL using high resolution scan – continuous measurement by DART-HRMS at 400 ºC and 0.6 mm/sec sample speed, consists of ammonium adduct $[M+NH_4]^+$ and protonated $[M+H]^+$ ions.

Table 7. DART-HRMS repeatability test of TTLs in high resolution scan with continuous measurement at 400 ºC and 0.6 mm/sec sample speed.

Analyte	Area ratio ± SD	RSD (%)
G-A	10.228 ± 2.36	23.1
G-B	3.357 ± 0.93	27.6
G-C	1.765 ± 0.63	35.9
BB	0.361 ± 0.04	9.8

*n = 10; SD = standard deviation
RSD = relative standard deviation

3.7 FI-HRMS analysis of terpene trilactones

During the positive ionisation using electrospray, the highest abundance of species formed was ammonium adduct $[M+NH_4]^+$ ion. Its abundance was followed by the formation of $[M+Na]^+$ species. Besides sodium and ammonium adducts, protonated molecule was observed at much lower abundance compared to them. In particular bilobalide, has no ammonium or sodium adduct ion. Typical ions detected during positive ionisation electrospray can be seen in Table 8 with the spectrum showed in Figure 8.

Table 8. Typical ions detected for terpene trilactones and picrotin using FI-HRMS in positive ionisation mode.

	G-A	G-B	G-C	BB	IS
Molecular mass (Da)	408	424	440	326	310
$[M+Na]^+$	431 (moderate)	447 (low)	463 (low)	349 (n.d.)	333 (n.d.)
$[M+NH_4]^+$	426 (high)	442 (low)	458 (low)	344 (n.d.)	328 (low)
$[M+H]^+$	409 (low)	425 (n.d.)	441 (low)	327 (n.d.)	311 (n.d.)

high = 50–100% abundance
moderate = 25–50% abundance
low = 5–25% abundance
n.d = not detectable (<5% abundance)

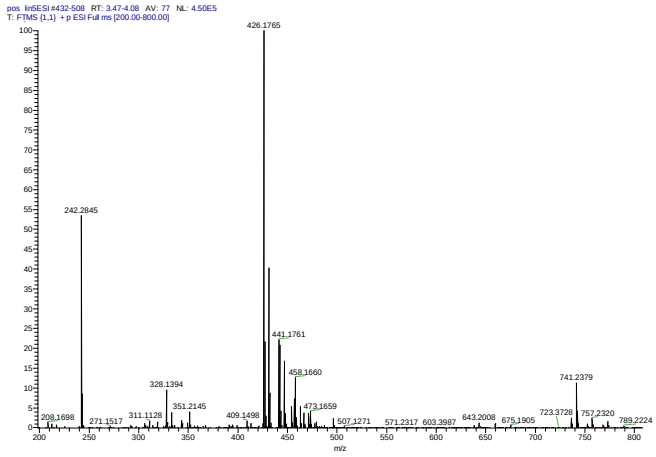


Figure 8. Typical extracted ion mass spectra of terpene trilactones and picrotin of 5 µg/mL pure ginkgolides mixture obtained by FI-HRMS in positive ionisation mode.

The abundant $[M+NH_4]^+$ ion as the most stable ion product during the positive ionisation was investigated as the marker ion for sample quantification. However, the relative amount of this ion compared with $[M+Na]^+$ and $[M+H]^+$ were variable depending on the trace amounts of those ions in the solution. In addition, bilobalide did not produce this ion. Therefore, the investigation of the usable ion for quantification was followed by the application of negative ion mode electrospray.

Negative ion electrospray produced abundant deprotonated ion $[M-H]^-$ for G-B, G-C, BB and IS. It becomes the base peak for each mass spectrum. This ion corresponds to the elimination of one proton during the fragmentation. Meanwhile, G-A did not produce this deprotonated ion.

Further fragmentation of G-B and G-C was the cleavage of fragment ion with mass 56 Da related to tert-butyl molecule (Van Beek, 2005). Furthermore, it is possible for one molecule of carbon monoxide to leave the structure due to the highly strained structures of lactone carbonyl groups of the A and C rings. This condition also leads to the loss of one molecule of neutral carbon dioxide even it is much lower compared with the fragmentation of neutral carbon monoxide (Sun et al., 2005). Typical ions formed during negative ion electrospray are depicted in Table 9 with the spectrum showed in Figure 9.

Table 9. Typical ions detected for terpene trilactones and picrotin using FI-HRMS in negative ionisation mode.

	G-A	**G-B**	**G-C**	**B-B**	**IS**
Molecular mass (Da)	408	424	440	326	310
$[M-H]^-$	407 (n.d.)	423 (high)	439 (high)	325 (moderate)	309 (high)
$[M-H]^-$ - tert-butyl	351 (n.d.)	367 (high)	383 (moderate)	-	-
$[M-H]^-$ - CO	379 (n.d.)	395 (low)	411 (n.d.)	-	-
$[M-H]^-$ - $2CO_2$	-	-	-	237	

high = 50–100% abundance
moderate = 25–50% abundance
low = 5–25% abundance
n.d = not detectable (<5% abundance)

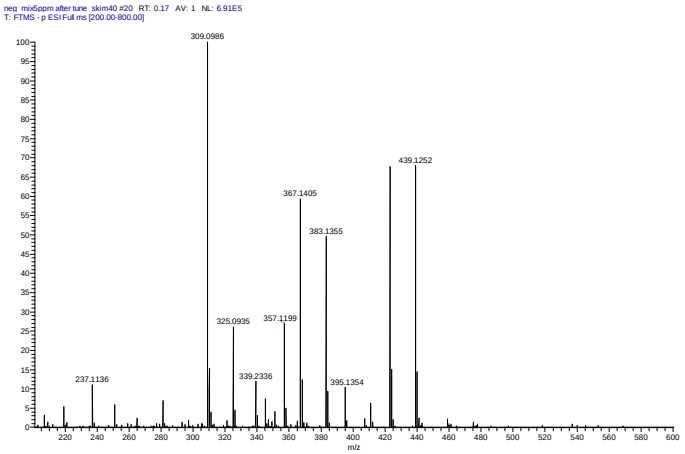


Figure 9. Typical mass spectrum of terpene trilactones and picrotin of 5 µg/mL pure ginkgolides mixture obtained by FI-HRMS in negative ionisation mode.

In particular for BB, more fragmentations were happening related to the elimination of a certain number of carbon dioxide molecules. The cleavage of this molecule is due to the less flexibility of its carbon skeleton compared with ginkgolides structures. Thus, BB gives a fragment of m/z 237 instead of its deprotonated form at m/z 325 as the loss of two carbon dioxide formed ions (Sun et al., 2005).

During the positive mode, there are two specific species that can be established as quantitation ion, whether sodium adducts or ammonium adduct ion. Typical fragment ion in high abundant was in ammonium adduct for G-A, G-B, G-C and IS; while BB has undetectable sodium and ammonium adduct ions. In order to focus on the highest abundance of fragment ion in the positive ion mode, ammonium chloride (NH_4Cl) was added into the TTLs mixture. Different amount of NH_4Cl added into the solution was optimised with 2, 4 and 6 mM in concentration. They gave different effect in enhancing the formation of ammonium adducts ion in 5 ppm mixture solution. Their abundance result can be seen in Table 10.

Table 10. Ammonium adduct ion abundance of FI-HRMS positive ionisation mode after the addition of NH_4Cl.

NH_4Cl addition (mM)	**Abundance**				
	G-A	**G-B**	**G-C**	**BB**	**IS**
2	7.29×10^5	5.11×10^5	3.60×10^5	1.23×10^5	4.40×10^5
4	1.65×10^6	1.35×10^6	9.11×10^5	3.99×10^5	1.08×10^6
6	6.41×10^5	6.74×10^5	4.62×10^5	2.27×10^5	6.09×10^5

The addition of 4 mM NH_4Cl resulted in the highest abundance of ammonium adduct and suppressed sodium adduct fragment ion as can be seen from Table 11 and its spectra in Figure 10.

Table 11. Typical ions detected for terpene trilactones using FI-HRMS in positive ionisation mode after the addition of 4 mM NH_4Cl.

	G-A	**G-B**	**G-C**	**BB**	**IS**
Molecular mass (Da)	408	424	440	326	310
$[M+Na]^+$	431 (n.d.)	447 (n.d.)	463 (n.d.)	349 (n.d.)	333 (n.d.)
$[M+NH_4]^+$	426 (high)	442 (high)	458 (high)	344 (moderate)	328 (high)
$[M+H]^+$	409 (low)	425 (n.d.)	441 (n.d.)	327 (low)	311 (n.d.)

high = 50–100% abundance
moderate = 25–50% abundance
low = 5–25% abundance
n.d = not detectable (<5% abundance)

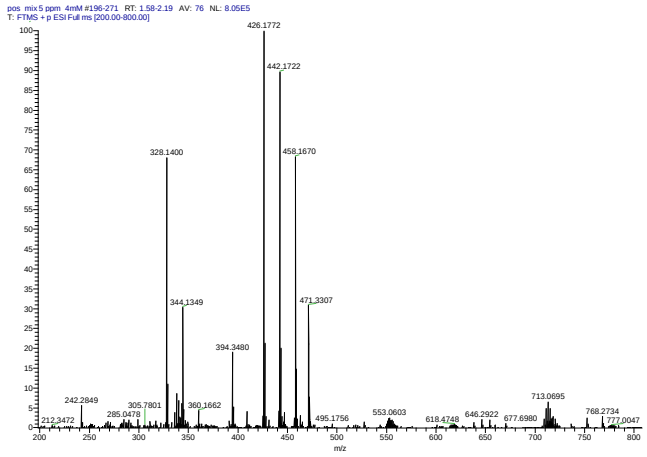


Figure 10. Typical mass spectrum of terpene trilactones and picrotin of 5 µg/mL pure ginkgolides mixture obtained by FI-HRMS in positive ionisation mode after the addition of 4 mM ammonium chloride.

3.8 FI-HRMS repeatability test

The repeatability test was performed by a series of eight times measurement using a 55 µg/mL mixture standard solution with the addition of 4 mM NH_4Cl. Based on the ion abundance optimisation, selective ion used for quantitation was ammonium adduct $[M+NH_4]^+$. For instance, G-C with a mass of 440 Da has a m/z 458 Da as the quantitation species ion (Figure 11). Further, the area of this selective ion chromatogram has been used in RSDs calculation. G-C area ratio was obtained by dividing the area of $[GC+NH_4]^+$ ion by the area of $[Pic+NH_4]^+$ ion. The repeatability test result can be seen in Table 12.

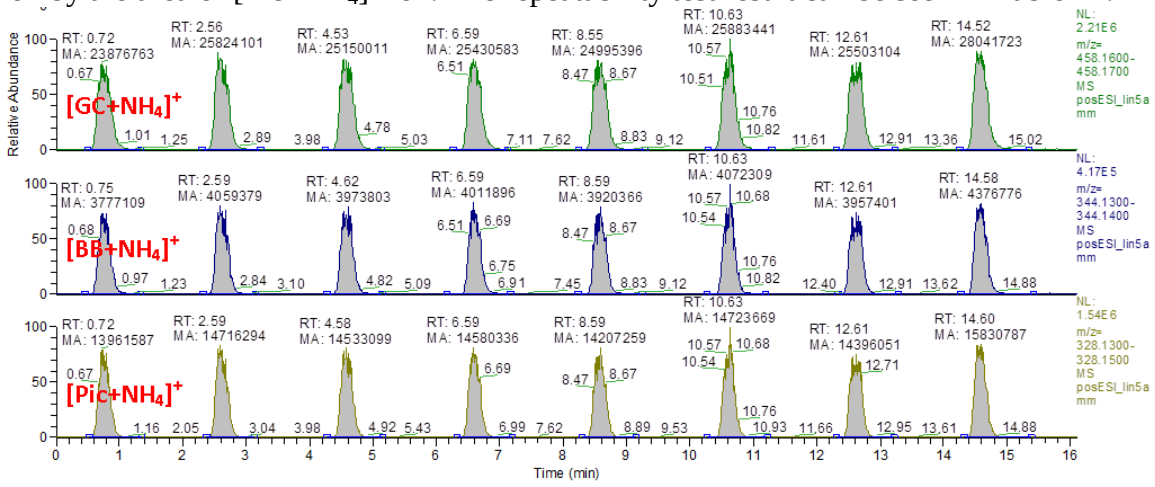


Figure 11. Extracted ion chromatograms of ginkgolides B, C and picrotin 25 µg/mL using FI-HRMS high resolution scan with a flow rate of 250 µL/min, consists of ammonium adduct $[M+NH_4]^+$ ion.

Table 12. FI-HRMS repeatability test of TTLs in high resolution scan.

Analyte	Area ratio ± SD	RSD (%)
G-A	5.321 ± 0.07	1.4
G-B	2.469 ± 0.02	0.8
G-C	1.750 ± 0.02	1.2
BB	0.275 ± 0.00	0.7

*n = 10; SD = standard deviation
RSD = relative standard deviation

RSDs values obtained from FI-HRMS reproducibility fulfilled the requirement for quantitative work. Therefore, linearity test was performed to see the performance of mass spectrometer detector related to a proportional response in accordance with the analyte concentration.

3.9 FI-HRMS linearity test

In order to see the performance of mass spectrometer detector used in FI-HRMS method, reproducibility test was performed using a series of points of standard solutions with the range of 2.5–70 µg/mL for G-A, G-B and G-C, while for BB it has the range of 10–140 µg/mL. Detector response of mass spectrometer followed linear equation that was given by y = ax + b where y is peak area ratio and x is sample concentration. Linear ranges and correlation coefficient for each TTLs are given in Table 13 and detailed graph calibration curves is attached in Appendix 3.

Table 13. Linear ranges and correlation coefficients of TTLs (r^2) from FI-HRMS linearity test.

Compounds	Linear range (µg/mL)	Calibration curve	Correlation coefficient (r^2)
G-A	2.5 – 70	y = 0.13909x + 0.09888	0.99925
G-B	2.5 – 70	y = 0.05867x + 0.03006	0.99678
G-C	2.5 – 70	y = 0.03535x + 0.05431	0.99434
BB	10 – 120	y = 0.00579x + 0.02680	0.99838

3.10 Relative recovery: liquid-liquid extraction evaluation of terpenoid compounds

To see the performance of sample clean-up process, relative recovery evaluation was done towards a mixture of pure ginkgolides using both UHPLC-ELSD and FI-HRMS assays. A certain concentration of known pure ginkgolides was proceeded using sample clean-up protocol. The result then was compared to those from methanol extraction without sample clean-up process. The relative recovery result by UHPLC-ELSD and FI-HRMS assay is provided in Table 14.

Table 14. Relative recovery results of terpene trilactones and picrotin by UHPLC-ELSD and FI-HRMS.

Analyte	Relative recovery (%)	
	UHPLC-ELSD	FI-HRMS
G-A	96.34	103.10
G-B	94.08	102.62
G-C	98.23	100.24
BB	99.14	104.47
Picrotin	97.66	99.70

As can be seen from Table 14, relative recovery results of FI-HRMS were higher than those from UHPLC-ELSD. In general, they were above 100% for the recovery. These values are acceptable in which they ranged within ± 5%.

3.11 Quantitative analysis of terpene trilactones

The main aim of this project is to evaluate the performance of the developing mass spectrometry method for both determination and direct quantification of terpene trilactones in *Ginkgo biloba* extract. Thus, quantitation work becomes more important to prove that this method is available for direct quantitation of terpenoid compounds. The

data of terpenoid contents from UHPLC-ELSD assay will serve as a benchmark. The quantitation work evaluation involved two important steps:

1. Accuracy calculation of a known NMR amount enriched ginkgolides after being analysed by UHPLC-ELSD and FI-HRMS.
2. Quantitation performance comparison between UHPLC-ELSD and FI-HRMS using *t*-test analysis, by comparing the analytical results of TTLs delivering by both methods.

Sample used for quantitation analysis were enriched ginkgolides and *Ginkgo biloba* crude extract. The former sample contains known amount of terpenoid compound which has been assayed by NMR method, and the latter is *Ginkgo biloba* extract that is commercially available as raw material in producing *Ginkgo biloba* preparations. Crude extracts consists of three different extracts, Japan GBE, Finzelberg Ekstrakte–4 and Finzelberg Ekstrakte–5. However, due to the unavailability of the Exactive instrument, analytical result of Japan GBE only obtained from UHPLC-ELSD measurement. Thus, this sample was not taken into account for the quantitation work evaluation of FI-HRMS method.

Quantitation work employed consists of two methods, using internal standard method and standard addition method.

3.11.1 Internal standard method

Calibration curve solutions used were the solutions, which contain the same amount of internal standard picrotin as has been added into the sample solutions. Samples were analysed in triplicate using both chromatography and mass spectrometry methods. The analytical results are provided in Table 15, shows the quantitation work using methanol extract and liquid-liquid extract samples.

G-J as a minor compound in the enriched ginkgolides sample can be determined by UHPLC-ELSD assay using the nearest terpenoid calibration curve, G-C.

3.11.2 Standard addition method

Standard addition method involved one level of spiked sample solution as a benchmark to calculate the content of terpenoid compounds in the samples. Sample used was the enriched gingkolides and result is provided in Table 16.

Table 15. Analytical results for terpene trilactones using internal standard method by UHPLC-ELSD and FI-HRMS.

Sample	TTLs	UHPLC-ELSD				FI-HRMS				NMR data (%)
		MeOH extract		LL extract		MeOH extract		LL extract		
		Content (% w/w ± SD)	RSD (%)	Content (% w/w ± SD)	RSD (%)	Content (% w/w ± SD)	RSD (%)	Content (% w/w ± SD)	RSD (%)	
Enriched ginkgolides	G-A	25.16 ± 0.36	1.4	20.89 ± 0.31	1.5	26.09 ± 0.09	0.3	23.91 ± 0.37	1.5	27.85
	G-B	10.41 ± 0.42	4.0	9.23 ± 0.16	1.7	14.57 ± 0.08	0.5	14.62 ± 0.14	0.9	11.92
	G-C	4.34 ± 0.17	4.0	4.18 ± 0.12	2.8	6.24 ± 0.07	1.1	6.94 ± 0.02	0.3	5.5
	BB	46.39 ± 0.42	0.9	46.95 ± 0.33	0.7	44.58 ± 0.75	1.7	51.42 ± 0.48	0.9	47.92
	G-J	0.94 ± 0.04	4.2	0.89 ± 0.07	7.8	-	-	-	-	0.94
Finzelberg Extrakte-4	G-A	-	-	0.88 ± 0.04	5.1	1.17 ± 0.03	2.6	-	-	-
	G-B	-	-	0.54 ± 0.04	8.3	0.96 ± 0.06	5.9	-	-	-
	G-C	-	-	0.85 ± 0.04	5.1	0.64 ± 0.04	6.1	-	-	-
	BB	-	-	2.58 ± 0.12	4.6	2.60 ± 0.13	4.9	-	-	-
Finzelberg Extrakte-5	G-A	-	-	0.85 ± 0.07	8.3	1.11 ± 0.01	1.2	-	-	-
	G-B	-	-	0.53 ± 0.00	0.3	0.85 ± 0.02	2.6	-	-	-
	G-C	-	-	0.78 ± 0.04	5.4	0.56 ± 0.02	2.8	-	-	-
	BB	-	-	2.31 ± 0.15	6.7	2.33 ± 0.03	1.5	-	-	-

*Analysis: intra-day (n = 3)
SD = standard deviation; RSD = relative standard deviation
MeOH extract = sample solution from methanol extraction
LL extract = sample solution after liquid-liquid extraction

Table 16. Analytical results for terpene trilactones using standard addition method by UHPLC-ELSD and FI-HRMS.

Sample	TTLs	UHPLC-ELSD				FI-HRMS				NMR data (%)
		MeOH extract		LL extract		MeOH extract		LL extract		
		Content (% w/w ± SD)	RSD (%)	Content (% w/w ± SD)	RSD (%)	Content (% w/w ± SD)	RSD (%)	Content (% w/w ± SD)	RSD (%)	
Enriched ginkgolides	G-A	22.80 ± 0.06	1.4	-	-	22.30 ± 0.29	3.4	-	-	27.85
	G-B	9.74 ± 0.03	4.0	-	-	12.41 ± 0.14	6.2	-	-	11.92
	G-C	4.04 ± 0.03	4.0	-	-	5.54 ± 0.05	7.7	-	-	5.5
	BB	43.26 ± 0.14	0.9	-	-	46.49 ± 0.03	6.0	-	-	47.92

*Analysis: intra-day (n = 3)

3.11.3 Calibration curve composition evaluation

BB content in enriched ginkgolides was observed depends on the terpenoid composition in the calibration curve solutions. Table 17 shows the difference in intercept value of BB calibration curve. Thus, it produced significant difference amount of BB during the analytical work of enriched ginkgolides. This result is due to the presence of ion suppression phenomenon since this method was carried out without compound separation.

Table 17. Calibration curve composition evaluation to determine the possibility of ion suppression phenomenon in TTLs direct quantitation using FI-HRMS.

Level	Standard concentration (µg/mL)		Equation	r^2	Methanol extract				Liquid-liquid extract				NMR data (%)
	GA/GB/GC	BB				Content (%)	SD	RSD (%)		Content (%)	SD	RSD (%)	
1	2.5	5	GA : y = 0.16580x - 0.12816	0.99934	GA	26.47	0.12	0.5	GA	23.07	0.49	2.1	27.85
2	5	10	GB : y = 0.07713x + 0.10311	0.99697	GB	12.87	0.09	0.7	GB	12.17	0.12	1.0	11.92
3	10	20	GC : y = 0.04437x + 0.09000	0.99619	GC	5.17	0.00	0.1	GC	5.43	0.02	0.4	5.50
4	25	50	BB : y = 0.00436x + 0.02261	0.99562	BB	56.45	0.47	0.8	BB	62.56	0.25	0.4	47.92
5	40	80											
6	55	110											
7	70	140											
1	2.5	7.5	GA : y = 0.14916x + 0.03581	0.99980	GA	28.65	0.13	0.5	GA	24.88	0.55	2.2	27.85
2	5	15	GB : y = 0.07039x + 0.16036	0.99709	GB	13.53	0.09	0.7	GB	12.76	0.13	1.1	11.92
3	10	30	GC : y = 0.04132x + 0.12948	0.99554	GC	4.88	0.00	0.1	GC	5.16	0.03	0.5	5.50
4	25	75	BB : y = 0.00459x + 0.03010	0.99816	BB	52.48	0.44	0.8	BB	58.28	0.24	0.4	47.92
5	40	120											
6	55	165											
7	70	210											
1	2.5	10	GA : y = 0.14080x + 0.09153	0.99871	GA	30.07	0.13	0.4	GA	26.07	0.58	2.2	27.85
2	5	20	GB : y = 0.06711x + 0.17478	0.99403	GB	14.05	0.09	0.7	GB	13.23	0.14	1.1	11.92
3	10	40	GC : y = 0.04000x + 0.13122	0.99365	GC	5.01	0.00	0.1	GC	5.30	0.03	0.5	5.50
4	25	100	BB : y = 0.00476x + 0.04460	0.99517	BB	48.48	0.40	0.8	BB	54.06	0.24	0.4	47.92
5	40	160											
6	55	220											
7	70	280											
1	2.5	12.5	GA : y = 0.13322x + 0.16243	0.99838	GA	31.41	0.14	0.4	GA	27.18	0.62	2.3	27.85
2	5	25	GB : y = 0.06401x + 0.21198	0.99033	GB	14.32	0.10	0.7	GB	13.47	0.15	1.1	11.92
3	10	50	GC : y = 0.03864x + 0.15843	0.98891	GC	4.70	0.01	0.2	GC	5.00	0.03	0.6	5.50
4	25	125	BB : y = 0.00482x + 0.07060	0.99159	BB	44.10	0.35	0.8	BB	49.60	0.24	0.5	47.92
5	40	200											
6	55	275											
7	70	350											

3.11.4 Accuracy evaluation of FI-HRMS method

Accuracy becomes the most important parameter in evaluating the developed method to be applied as a quantitation method. In order to determine the accuracy performance, a sample which was measured with NMR was used as the benchmark. Thus, the analytical result of FI-HRMS and UHPLC-ELSD were compared to the NMR result as can be seen in Table 18. The accuracy value represents the deviation in percentage between the analytical result obtained by FI-HRMS and UHPLC-ELSD compared to the NMR assay. A low percentage in accuracy represents the high accuracy of the analytical result since it has small deviation result compared to the NMR data.

Table 18. Accuracy evaluation of FI-HRMS and UHPLC-ELSD methods based on the NMR analytical results of the enriched ginkgolides.

NMR data (%)	Accuracy (%) – Internal standard method				Accuracy (%) – Std. addition method	
	UHPLC-ELSD		FI-HRMS		UHPLC-ELSD	FI-HRMS
	MeOH extr.	LL extr.	MeOH extr.	LL extr.	MeOH extr.	MeOH extr.
G-A = 27.85	9.7	25.0	6.3	14.1	18.1	19.9
G-B = 11.92	12.7	22.6	22.2	22.7	18.3	4.1
G-C = 5.50	21.1	24.0	13.5	26.2	26.5	0.7
BB = 47.92	3.2	2.0	7.0	7.3	3.5	3.0

*MeOHextr. = sample solution from methanol extraction
LL extr. = sample solution after liquid-liquid extraction

Accuracy evaluation of the developed method, FI-HRMS, compared to UHPLC-ELSD as the benchmark method shows significant variation. From FI-HRMS data, it can be seen that the lowest accuracy was depicted by the highest deviation of the analytical result after extraction with internal standard method, 26.2%, compared to the actual content of G-C in the sample. On the other hand, the most accurate data was obtained by the quantitation of G-C using standard addition method without sample clean-up process, 0.7%.

From UHPLC-ELSD accuracy data, the less accurate data was performed by the analytical result of G-C by standard addition method, 26.5% in deviation. Meanwhile, the highest accuracy data was obtained from the quantitation of BB by internal standard method with 3.2% in deviation.

In general, FI-HRMS has higher accuracy than UHPLC-ELSD by standard addition method preparation. It is assumed that during the quantitation work, sample solution has the same environmental condition with those of solution contained reference compounds. Hence the deviations of the results are smaller compared to the quantitation work by internal standard method.

Mostly, the accuracy of BB is the highest compared to the other compounds both of UHPLC-ELSD and FI-HRMS methods.

3.11.5 Comparison between the analytical results of methanol extract and liquid-liquid extract sample

Besides accuracy evaluation, quantitation performance of the developed MS method also being determined by the comparison of quantitation work for methanol extract and liquid-liquid extract measurements. By this, the deviation of the analytical result with and without sample clean-up is taken into account to see the influence of liquid-liquid

extraction process into the quantitation performance of the methods. In this case, methanol extract sample become a benchmark to calculate the deviation. The result of the deviation can be seen in Table 19.

Table 19. The comparison between methanol extract and liquid-liquid extract analytical results using both UHPLC-ELSD and FI-HRMS methods.

	Content (%) – UHPLC-ELSD			Content (%) – FI-HRMS		
	MeOH extract	LL extract	Deviation (%)	MeOH extract	LL extract	Deviation (%)
G-A	25.16	20.89	17.0	26.09	23.91	8.4
G-B	10.41	9.23	11.3	14.57	14.62	0.3
G-C	4.34	4.18	3.7	6.24	6.94	11.2
BB	46.39	46.95	1.2	44.58	51.42	15.3

In order to see the difference between UHPLC and FI-HRMS performance in quantitation work, the deviation between these methods was evaluated in their analytical result performance as can be seen in Table 20.

Table 20. The comparison between UHPLC-ELSD and FI-HRMS analytical results in measuring methanol extract and liquid-liquid extract samples.

	Content (%) – MeOH extract			Content (%) – LL extract		
	UHPLC-ELSD	FI-HRMS	Deviation (%)	UHPLC-ELSD	FI-HRMS	Deviation (%)
G-A	25.16	26.09	3.7	20.89	23.91	14.5
G-B	10.41	14.57	40.0	9.23	14.62	58.4
G-C	4.34	6.24	43.8	4.18	6.94	66.0
BB	46.39	44.58	4.3	46.95	51.42	9.5

Based on the deviation calculation from Table 18 and 19, the deviation between UHPLC-ELSD and FI-HRMS analytical performance was higher than the deviation between methanol extract and liquid-liquid extract analytical result, with the highest deviation reached 66.0% in difference for the assay of liquid-liquid extract sample. The lowest deviation value was the comparison of G-B analytical result between methanol extract and liquid-liquid extract using FI-HRMS method.

3.11.6 Quantitation work performance: comparison between FI-HRMS and UHPLC-ELSD

Based on the quantitation assay using FI-HRMS and UHPLC-ELSD, there are some different values of the measured terpenoid compounds. These values then should be evaluated to see whether the developed method will have a performance as a quantitation method as the benchmark method. The comparison test employed is *t*-test, to compare the mean value of terpenoid content in the sample between FI-HRMS and UHPLC-ELSD results. Furthermore, *t*-value obtained was compared to *t*-table to make a decision, as provided in Table 21 and 22 (see Appendix 4 for calculation details).

Table 21. Quantitation work performance comparison between UHPLC-ELSD and FI-HRMS using internal standard method.

	UHPLC-ELSD		FI-HRMS		S^2	S	*t*-value	*t*-table (d.f=4, P=0.05)
	x_1	s_1	x_2	s_2				
1. Ginkgolides A								
Enriched ginkgolides								
a. Methanol extract	25.16	0.36	26.09	0.09	0.069	0.262	4.34	2.78
b. LL extract	20.89	0.31	23.91	0.37	0.117	0.341	10.84	
Finzelberg Extrakte-4	0.88	0.04	1.17	0.03	0.001	0.035	10.05	
Finzelberg Extrakte-5	0.85	0.07	1.11	0.01	0.003	0.050	6.37	
2. Ginkgolides B								
Enriched ginkgolides								
a. Methanol extract	10.41	0.42	14.57	0.08	0.091	0.302	16.85	2.78
b. LL extract	9.23	0.16	14.62	0.14	0.023	0.150	43.91	
Finzelberg Extrakte-4	0.54	0.04	0.96	0.06	0.003	0.051	10.09	
Finzelberg Extrakte-5	0.53	0.00	0.85	0.02	0.000	0.014	27.71	
3. Ginkgolides C								
Enriched ginkgolides								
a. Methanol extract	4.34	0.17	6.24	0.07	0.017	0.130	17.90	2.78
b. LL extract	4.18	0.12	6.94	0.02	0.007	0.086	39.30	
Finzelberg Extrakte-4	0.85	0.04	0.64	0.04	0.002	0.040	6.43	
Finzelberg Extrakte-5	0.78	0.04	0.56	0.02	0.001	0.032	8.52	
4. Bilobalide								
Enriched ginkgolides								
a. Methanol extract	46.39	0.42	44.58	0.75	0.369	0.608	3.65	2.78
b. LL extract	46.95	0.33	51.42	0.48	0.170	0.412	13.29	
Finzelberg Extrakte-4	2.58	0.12	2.60	0.13	0.016	0.125	0.20	
Finzelberg Extrakte-5	2.31	0.15	2.33	0.03	0.012	0.108	0.23	

x_1 & x_2 = mean value from UHPLC-ELSD and FI-HRMS assay
s_1 & s_2 = standard deviation from UHPLC-ELSD and FI-HRMS assay
S = pooled standard deviation
d.f = degree of freedom (3+3–2=4)

Table 22. Quantitation work performance comparison of between FI-HRMS and UHPLC-ELSD using standard addition method of the enriched ginkgolides in methanol extract.

	UHPLC-ELSD		FI-HRMS		S^2	S	*t*-value	*t*-table (d.f=4, P=0.05)
	x_1	s_1	x_2	s_2				
1. Ginkgolides A	22.80	0.06	22.30	0.29	0.044	0.209	2.92	2.78
2. Ginkgolides B	9.74	0.03	12.41	0.14	0.010	0.101	32.30	2.78
3. Ginkgolides C	4.04	0.03	5.54	0.05	0.002	0.041	44.56	2.78
4. Bilobalide	43.26	0.14	46.49	0.03	0.010	0.101	39.07	2.78

x_1 & x_2 = mean value from UHPLC-ELSD and FI-HRMS assay
s_1 & s_2 = standard deviation from UHPLC-ELSD and FI-HRMS assay
S = pooled standard deviation
d.f = degree of freedom (3+3–2=4)

3.12 FI-HRMS performance in terpene trilactone measurement compared to UHPLC-ELSD method

FI-HRMS analytical performance was evaluated based on the criterion established related to the obtained data from optimisation and quantitation work. Its performance then compared to those of UHPLC-ELSD to get the best method over the other ones. The developing mass spectrometry method has some characteristics as described in Table 23 (represented by one sample in triplicate preparation).

Table 23. Analytical performances of FI-HRMS and UHPLC-ELSD methods based on the optimisation and quantitation data obtained.

Criterion	UHPLC-ELSD	FI-HRMS
Accuracy	- 2.0 – 25.0% (internal std. method) - 3.5 – 26.5% (std. addition method)	- 6.3 – 26.2% (internal std. method) - 0.7 – 19.9% (std. addition method)
Time	- Sample clean-up process: 3 hours. - Instrument analysis: 3 hours (9 injections, 20 min analysis for one injection). - Data integration: in minutes.	- Sample extraction time: need further study (MeOH or LLE). - Instrument analysis: 13.5 min (9 injections, 1.5 min analysis for one injection). - Data integration: in minutes.
Cost	- Need sample clean-up prior to analysis (extra use of buffer 5% NaH_2PO_4, NaCl and Ethyl acetate). - Solvent used in instrument analysis (MeOH-THF-H_2O): 72 mL (3 hours, flow rate 0.4 mL/min).	- Sample extraction method: need further study (MeOH or LLE). - Solvent used in instrument analysis: pure methanol (extra use of NH_4Cl for modifier). - Total solvent used for one sample analysis: 3.375 mL (13.5 min, flow rate 250 µL/min).
Reproducibility	- RSDs of instrument performance: 1.8 – 3.0 %. - RSDs of quantitation work: 0.9 – 19.3% (int. std. method) and 0.9 – 5.0% (std. add. method)	- RSDs of instrument performance: 0.7 – 1.4 %. - RSDs of quantitation work: 0.3 – 5.9% (int. std. method) and 3.4 – 7.7% (std. add. method)
Specificity	- Determine terpene trilactones in good resolution. - The presence of G-J as a minor constituent can be expressed as well.	- Distinguish G-A, G-C and BB by their specific mass determination. - Not able to distinguish between G-B and G-J as a minor constituent since they have the same molecular mass. - BB content in enriched ginkgolides was observed depend on the terpenoid composition in the calibration curve due to ion suppression phenomenon.
Sensitivity	- Determine terpenoid compounds ≥ 25 µg/mL with good repeatability.	- Determine terpenoid compounds ≥ 2.5 µg/mL with good repeatability.

Chapter 4. Discussion

4.1 UHPLC-ELSD: instrument optimisation

UHPLC-ELSD is a powerful tool for detecting compounds which do not have UV absorbance properties like ginkgolides and bilobalide. Address to a former paper (van Beek and Montoro, 2009), it was mentioned that a composition of MeOH-THF-H_2O with approximately ratio of 20:10:70 was the best solvent for TTLs and resulted sophisticated resolution of G-A, G-B, G-C, G-J and bilobalide. However, when it was applied to run the sample with picrotin as the internal standard, picrotin and G-J were coeluted. Therefore, an optimisation using DryLab software was employed.

As the input data for the construction of solvent composition, we have to input raw data of retention times of chromatograms obtained from the individual solvent as the part of final solvent composition. Hence, analytes elution using MeOH, THF and MeOH–THF–water was established first. The main requirement for this method is that all of the analytes should be eluted within the gradient time. Thus, all of the compounds should be eluted within 6 and 18 minutes gradient time (Figure 2). To ascertain the retention time, single analyte solution was injected using the same gradient systems. Then, the data of their retention time will be submitted into the DryLab software.

Isocratic chromatography to separate the six compounds that was developed is more convenient and beneficial to the routine analysis in the quality control of products. When it is applied to a real extract, it is necessary to clean the column with 60% methanol within 5 min after the six compounds have been eluted to remove retained impurities from the column. After the column was re-equilibrated for 5 min with the isocratic solvent, it was ready for the next run. Totally, running time employed was 20 min with gradient chromatographic system.

Picrotin, BB, G-C, G-A and G-B was obtained with retention times of 3.15, 4.10, 4.53, 7.70 and 9.97 min respectively. In Figure 3C, a small peak between picrotin and BB corresponded to the peak of ginkgolide J (G-J) at a retention time of 3.65 min. However, due to the limitation of G-J standard, the peak could not be quantified. The other option is using the G-C calibration curve to estimate the content of G-J in the sample.

From the repeatability test employed to check the performance the instrument, RSDs values are within 1.8–3.0% for intra-day analysis, as depicted in Table 3. Furthermore, during the quantitation work, it has varied RSDs value from 0.9% to 19.3% for internal standard method preparation and 0.9–5.0% for standard addition method preparation (Table 15 and 16).

From the linearity test has been observed, all of the standard calibration curves showed linear response with r^2 values ≥ 0.99. Meanwhile, the response intensity of evaporative light-scattering detector was varied over different time measurements. Hence, it is necessary to run the standard solutions prior to sample quantitative work to ascertain that the response of sample measurements were proportional with the response of the standard solutions. Thus, we can ensure that the results of quantitation work represented the content of TTLs in the sample.

4.2 Liquid-liquid extraction of terpene trilactones

Liquid-liquid extraction process was done using 5% NaH_2PO_4 within the pH range of 3–5. The observed aqueous solution showed that the pH was 4.27. During the extraction, NaCl was added with a concentration of 20%. It was important to enhance the extraction of terpene trilactones into the organic phase and accelerate phase separation of the aqueous and organic phase. The addition of 20% THF was beneficial for a quantitative extraction of BB and G-J because it would increase the polarity of the organic phase.This sample clean-up procedure has been validated and gave recoveries values ≥92.9% depending on the components (Lang et al., 2004). Sample clean-up is important to eliminate some impurities that overlap with ginkgolides peak, resulted unreal content of analytes.

4.3 Liquid-liquid extraction evaluation of terpenoid compounds

Relative recovery values from both chromatography and mass spectrometry methods are above 90% with the range of 94.08–99.14% for UHPLC-ELSD and 99.7–104.47% for FI-HRMS (Table 14 and 15). It can be observed that all of the relative recovery values by FI-HRMS are higher than those of UHPLC-ELSD result, even more than 100% for those compounds except picrotin. By UHPLC-ELSD, peak area value was depending on precise peak integration. In particular for G-B, it has a broaden peak at the last eluting time. Thus, a small mistake in the peak integration (in defining baseline, for instance) will greatly affect the area value. Hence, G-B has the lowest recovery, 94.08%.

Relative recovery obtained by both UHPLC-ELSD and FI-HRMS also depends on the use a proper internal standard. Picrotin as the internal standard has identical chemical properties as the analytes and has good stability, recovery and free from ion suppression matters, showed by the high relative recovery values by both of these methods. The higher recovery value of picrotin by FI-HRMS assay, 99.70%, brings to high recovery values of TTLs compounds which were within an acceptable range, ± 5%.

The high relative recovery values indicates that sample clean-up process can be applied to eliminate some impurities as shown in Figure 5 and extract the terpenoid compounds into the organic solution used. The developed mass spectrometry method has been observed has high recovery in determining terpenoid compounds with UHPLC-ELSD as the benchmark.

4.4 DART-HRMS: instrument optimisation

4.4.1 Ionisation mode optimisation

In positive ionization mode, there are two typical ions for each terpenoid compound, observed as ammonium adduct $[M+NH_4]^+$ and protonated ion $[M+H]^+$. In order to focus on the formation of ammonium adduct ion, ammonium chloride was added as a modifier. The addition of 10 mM NH_4Cl was not effective in suppressing the formation of protonated ion during the measurement using positive mode. Furthermore, the relative abundance of $[M+NH_4]^+$ and $[M+H]^+$ species were variable within different calibration solutions tested. Therefore, the repeatability test was performed by considering the total amount of $[M+NH_4]^+$ and $[M+H]^+$ species from terpene trilactones and the internal standard picrotin.

In negative ion mode, there were no adducts and the typical mass ion obtained by SIM was deprotonated ion $[M-H]^-$. On the other hand, specific mass of G-A, G-B and G-C fragmented compounds were formed related to the loss of tert-butyl compound.

From Figure 6 it can be seen that the signal obtained was much stronger in positive mode (1.62×10^6) in spite of ammonium adducts and protonated ion released. On the other hand, Figure 5 shows that negative mode has less abundance ion (3.43×10^5) and resulted fragment ion as the cleavage of mass 56 m/z for all of the TTLs compounds. It corresponds to the loss of isobutane. In addition, in negative mode, G-A (m/z 407) has very low abundance. Therefore, positive ionisation was most suited for quantitative work.

4.4.2 DART-HRMS repeatability test

From Table 7 it can be seen that RSDs values of area ratio between terpene trilactones to the internal standard did not fulfil the requirement. It has a range within 9.8–35.9%. Therefore, the linearity test cannot be performed using this method and it cannot be applied for sample quantitation.

The high RSDs value represents the fluctuation in ratio area average between TTLs and IS in which the formation of $[M+NH_4]^+$ and $[M+H]^+$ species during the measurement was uneven. Uneven formation of these ions can be caused by systematic errors during the measurement. One possible reason is the ion affinity difference with the mesh surface of the analytes in a mixture. A mixture of compounds also allowed the presence of mutual ion interaction in the liquid phase that affects the formation of typical ions during the analysis. Uneven temperature from the DART stream cause uneven formation of the quantitation ions in which the evaporation and ionisation of the analytes were strongly influenced. Further, a constant DART stream gas flow is important in producing a constant rate of ion formation during the analysis (Harding et al., 2014).

Based on this, the investigation must be carried to see whether the non-reproducible data comes from the DART instrumentation or from the mass spectrometer. Hence, the instrumentation was changed into flow injection high resolution mass spectrometry (FI-HRMS). Mass spectrometer was employed as the detector, combined with methanol flow injection from liquid chromatography equipment without the use of column. Therefore, this modification in the instrumentation will give clear information about the mass spectrometer detector performance unless the main goal for the measurement has been changed.

4.5 FI-HRMS: instrument optimisation

4.5.1 Ionisation mode optimisation

From the presence of some fragment ions during the ionisation using positive mode (Table 8, Figure 8) and negative mode (Table 9, Figure 9), it was observed that there were more fragment ions formed during negative ion mode. In addition, the abundance of G-A in negative ion mode was not detectable. On the other hand, ammonium or sodium adduct ion for bilobalide in positive ionisation were not formed. Therefore, the addition of a certain modifier to enhance specific ion formation as a marker in quantitation work becomes crucial. The influence of modifier addition only can be investigated in positive ion mode. Thus, ammonium chloride (NH_4Cl) was added into the TTLs mixture to

enhance the abundance of ammonium adduct ion. It was observed that the concentration of 4 mM of NH_4Cl gave the highest abundance of ammonium adduct ion and suppressed sodium adduct fragment ion (Table 10, Figure 10). Therefore, this modifier then was added into the calibration solutions and sample solutions to enhance the formation of ammonium adduct fragment ion.

4.5.2 FI-HRMS repeatability and linearity

Based on the observed result related to repetition measurement, it can be seen that FI-HRMS was reproducible for direct quantitation of *Ginkgo biloba* extract with RSD values 0.7–1.4 % (Table 12). Compared to UHPLC-ELSD, the developed mass spectrometry method has better reproducibility during quantitation work. By internal standard method, the RSDs values are within the range of 0.3–5.9% (Table 15), whereas using standard addition method the RSDs are varies from 3.4% to 7.7% (Table 16). Furthermore, the mass detector response has a linear equation that with correlation coefficients values ≥ 0.99 (Table 13).

A reproducible and linear response of MS detector during the analysis using FI-HRMS is quite promising to exploit this method for direct quantitation of TTLs in *Ginkgo biloba* extracts. However, the most important parameter to see whether a method can be applied to quantify analytes in the sample is how accurate this method in measuring a known amount of compound compared to the real content. Thus, an enriched ginkgolides that have been measured using NMR become a tool in the accuracy evaluation of the developed method.

4.6 Quantitative analysis or terpene trilactones in *Ginkgo biloba* extracts

Based on the quantitation result, it was observed that mostly there were some significant differences in the terpenoid content between UHPLC-ELSD and FI-HRMS assays. These results related with ion suppression phenomenon of terpene trilactones in the mixture. It is supported by the data of bilobalide composition evaluation in pure ginkgolides (Table 7). It was observed that the difference of bilobalide composition in the mixture can greatly affect the intercept value of the calibration curve. Further, it influenced the measured content of bilobalide in the sample.

Ion suppression is one of important factors that can affect the quantitative performance of a mass detector. Analyzed by injection (no analytical column) brings to the presence of coeluted analytes ultimate to the mass detector. Smaller analyte or more polar analyte is more susceptible from ion suppression incident (Annesley, 2003). In this case, bilobalide which is more polar than the other compounds and has smaller molecular size suffers from this phenomenon.

Besides bilobalide, G-B content by FI-HRMS assay also found to be higher than by UHPLC assay. It is because the samples also contain G-J which has the same mass as G-B, 424 Da., and the developing mass spectrometry method was not able to distinguish them. It contains 0.94% of G-J according to the NMR assay. This drawback has a relation with the inability of the system in select a specific mass since this system was not using column for the analyte separation

As can be seen in Table 15, methanol extract of FI-HRMS contained 14.57% of G-B, while after measuring with UHPLC-ELSD it contained 10.41% of G-B. It was found that the sample also contain G-J 0.94% after measuring with UHPLC-ELSD method.

Based on the comparative data between UHPLC-ELSD and FI-HRMS analytical performance in measuring the content of TTLs in both of methanol extract and liquid-liquid extract, it is concluded that the quantitation performance of FI-HRMS was significantly different compared to those of UHPLC-ELSD, with the highest deviation was about 66% for G-C determination after sample clean-up was performed. It is followed by the deviation of G-B quantitation after liquid-liquid extraction, 58.4% (Table 20).

For methanol extract measurement, FI-HRMS and UHPLC also showed a significant deviation in which for the determination of G-C the deviation was 43.8%, followed by the deviation of G-B measurement, 40.0%.

4.7 Comparison between UHPLC-ELSD and FI-HRMS performance in quantitation work of terpene trilactones: *t*-test analysis

Based on the evaluation of quantitation work performance for both UHPLC-ELSD and FI-HRMS, it was found that these methods have significant difference in quantifying ginkgolides and bilobalide. Almost all of the quantitation work results have *t*-value more than *t*-table, 2.78. Only bilobalide content of Finzelberg Extrakte–4 and Finzelberg Extrakte–5 that have the same result between UHPLC-ELSD and FI-HRMS with *t*-value lower than 2.78; 0.20 and 0.23 respectively (Table 21).

By this result, it can be observed that the ability of FI-HRMS in quantifying terpenoid contents in *Ginkgo biloba* extract was not equal with the benchmark method, UHPLC-ELSD. However, it is still questionable whether the former method or the latter one which has a superior position for this work. Further quantitation work with self-prepared enriched ginkgolides samples should be delivered using NMR to get clearer conclusion about the method performance in this field.

4.8 FI-HRMS performance in terpene trilactones determination compared to UHPLC-ELSD

A comparison of the developing mass spectrometry method to UHPLC-ELSD method was based on six criteria; accuracy, time, cost, reproducibility, specificity and sensitivity. In this comparison, it was clearly shown that MS based method has better analysis time and reduced cost (per sample) with high reproducibility that is important in supporting sample quantitation work. In addition, FI-HRMS was more sensitive than UHPLC-ELSD which means that it is applicable to detect terpene trilactones in lower concentration level and available to determine the adulteration of *Ginkgo biloba* product with another kind of herbal plant raw material. However, its specificity was fair since it was not able to distinguish between G-B and G-J which have the same molecular mass, 242 Da.

The developed MS method also has a limitation in terms of accuracy. Based on the comparison of analytical results between FI-HRMS and NMR assay, the accuracy were ranged within 0.7–26.2%, which shows that the obtained measured compounds are varied

from different ginkgolides that were influenced by ion suppression among the compounds due to the interaction of the compounds in the mixture.

While there is a lack in specificity and accuracy, FI-HRMS offers some advantages. This method excels in time needed for analysis whether for sample preparation or during instrument analysis. It gives some benefits when this method is applied in a quality control laboratories as a part of In Process Control (IPC) activity during the production of *Ginkgo biloba* extract in a company, or even if it is used in an authority institution laboratory such as Food and Drug Administration Agency. More samples marketed can be covered for their quality control management in a fast analysis time.

In a relation with the time used in the analysis, the cost needed for the analysis can be reduced as well. It has a benefit in the efficiency of chemicals used during the machine measurement. By the using of UHPLC-ELSD, one sample can be finished in 3 hours with a solvent flow rate of 0.4 mL/min, to get a complete result for terpene trilactones quantitation. Meanwhile, FI-HRMS only need 13.5 minutes to quantify one sample with a flow rate of 250 uL/min methanol. Hence, during the analysis, total solvent consumed for UHPLC-ELSD to analyse one sample was 72 mL, while FI-HRMS only need 4.5 mL of methanol. However, the possibility of no sample clean-up procedure prior to analysis by FI-HRMS still need to be evaluated since the quantitation work was influenced by ion suppression phenomenon due to the absence of compound separation.

Another advantage of the developed method is the high reproducibility of this method. This method was equipped with a loop system in the injector system that can ensure the same volume of sample that can reach the detector. Therefore, it is not difficult to reach a reproducible data during the analysis. Reproducible data is very important for quantitative analysis since quantitation work always need repetition of the sample measurements.

Furthermore, the proposed method has a better sensitivity for sample measurement. It has a benefit to determine terpene trilactones lower concentration level and available to determine the adulteration of *Ginkgo biloba* product with another kind of herbal plant raw material.

Conclusion

Based on the weighted parameters, it is concluded that FI-HRMS is generally more beneficial over UHPLC-ELSD method prior to the fast and simple measuring system. Its excellence is based on the time, cost and reproducibility criteria that have been evaluated compared to those of UHPLC-ELSD method. Besides that, FI-HRMS is more sensitive in determining TTLs. However, this method also has a drawback in terms of specificity due to its inability in distinguishing G-B and G-J in the solution. Furthermore, it lacks in accuracy related to the analytical results of terpene trilactones in the enriched ginkgolides. The quantitation work of FI-HRMS is still questionable since it has significant difference compared to the result of UHPLC-ELSD and NMR assay

Future research

The absence of sample clean-up process prior to analysis becomes an opportunity to be explored further. It is important since the developed method has a specific feature in terms of direct MS determination and quantitation analysis of TTLs in Ginkgo biloba extract. It will be an excel point of this method in which low amount of chemicals used and less time spent are more favourable. Besides that, the simple sample preparation can increase the efficiency with less labour procedure. Thus, it becomes a more favourable method to control the quality of *Ginkgo biloba* extract.

Analytical result using self-prepared sample by NMR should be performed further to confirm the analytical result of TTLs using FI-HRMS. It is based on the reason that the confirmation using UHPLC-ELSD as the benchmark also result uncertain data correlated with the accuracy of both of the evaluated methods.

A simple sample preparation and instrumentation further can be applied to see the profile of total compounds contained in *Ginkgo biloba* extract. By this, total spectrum of the sample can be evaluated with the address of specific mass of ginkgolides, ginkgolic acids, flavonoids or other important compound inside the sample. Hence, this method will be applicable for *Ginkgo biloba* profile to support the quality control mapping of *Ginkgo biloba* based on the specific mass compounds inside.

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Appendix 1. Retention time confirmation for DryLab application.

1. Solvent: H_2O – 100% MeOH

No.	Time gradient	Temperature (°C)	Solvent gradient	Retention time (min.)					
				Bilobalide	Picrotin	G-J	G-C	G-A	G-B
1	6 min.	25	5 - 95% MeOH	4.852	4.841	4.915	5.001	5.514	5.593
2	18 min.	25	5 - 95% MeOH	8.018	8.221	8.776	9.020	10.496	10.739
3	6 min.	50	5 - 95% MeOH	4.490	4.490	4.490	4.690	5.228	5.228
4	18 min.	50	5 - 95% MeOH	6.970	7.205	7.863	8.146	9.600	9.848

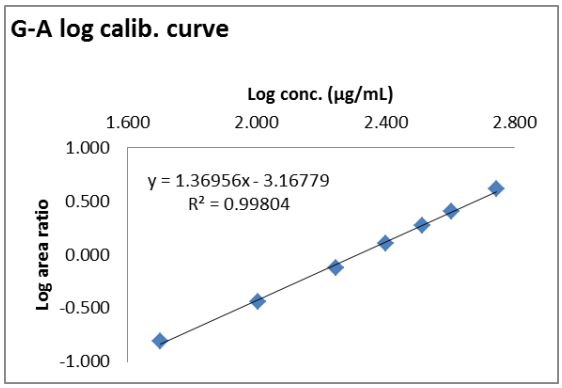
2. Solvent: H_2O – 80% THF

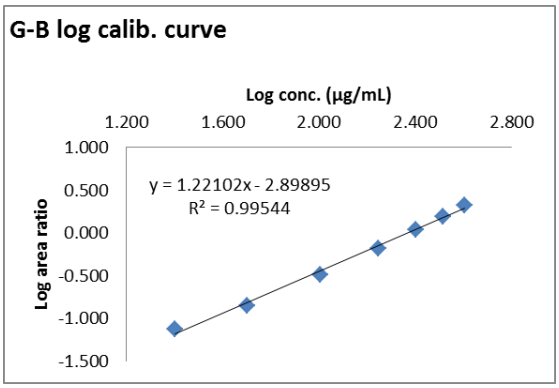
No.	Time gradient	Temperature (°C)	Solvent gradient	Retention time (min.)					
				Picrotin	G-J	G-C	G-A	Bilobalide	G-B
1	6 min.	25	5 - 95% THF	4.749	4.749	5.210	5.420	5.525	5.670
2	18 min.	25	5 - 95% THF	7.310	7.561	8.743	9.661	9.661	10.614
3	6 min	50	5 - 95% THF	4.399	4.399	4.834	5.170	5.170	5.338
				Picrotin	G-J	G-C	Bilobalide	G-A	G-B
4	18 min	50	5 - 95% THF	6.049	6.313	7.275	8.120	8.262	9.238

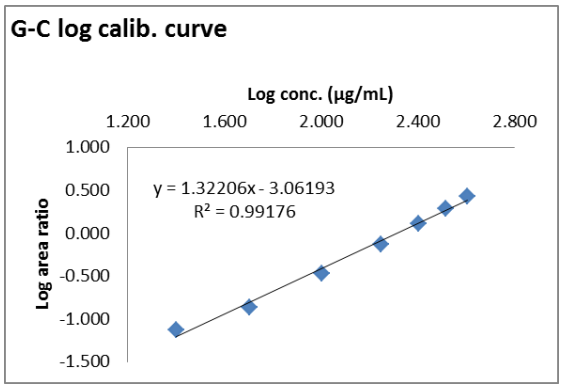
3. Solvent: H_2O – (50% MeOH - 40% THF - 10% H_2O)

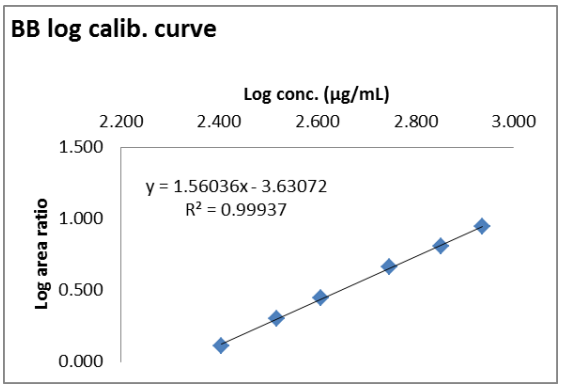
No.	Time gradient	Temperature (°C)	Solvent gradient	Retention time (min.)					
				Picrotin	G-J	G-C	Bilobalide	G-A	G-B
1	6 min.	25	5 - 95% (MeOH-THF-H_2O)	4.762	4.762	5.060	5.320	5.320	5.607
2	18 min.	25	5 - 95% (MeOH-THF-H_2O)	7.425	7.657	8.456	8.820	9.486	10.349
3	6 min.	50	5 - 95% (MeOH-THF-H_2O)	4.270	4.270	4.562	4.766	4.766	5.136
4	18 min.	50	5 - 95% (MeOH-THF-H_2O)	6.219	6.509	7.161	7.342	8.231	8.976

Appendix 2. UHPLC-ELSD calibration curves.

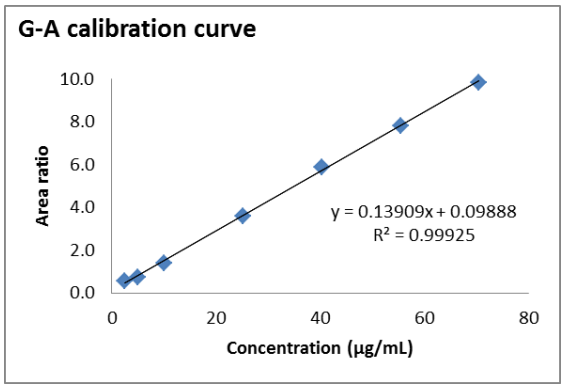


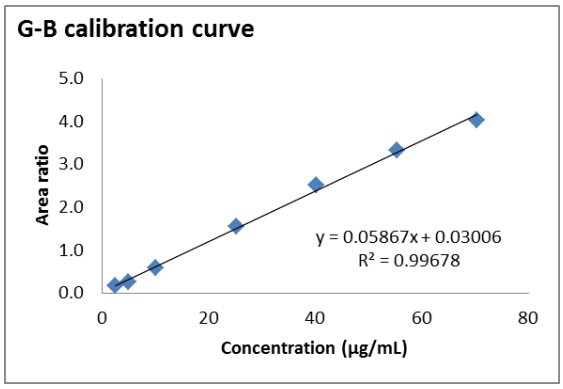


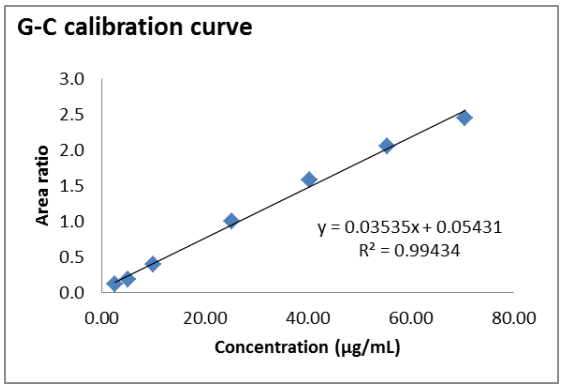


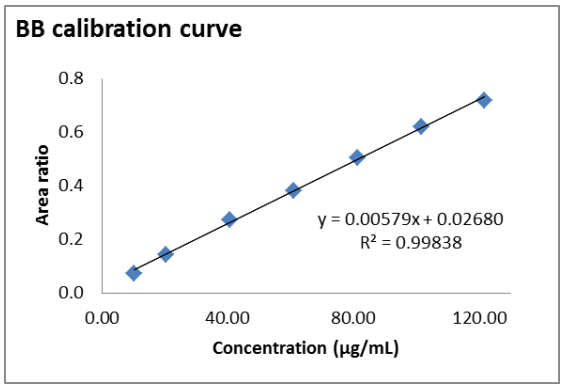


Appendix 3. FI-HRMS calibration curves.









Appendix 4. *t*-test calculation of UHPLC-ELSD and FI-HRMS in ginkgo terpene trilactones analytical performance comparison.

a. Case data.

Data used for calculation example was obtained from Table 16, related to analytical result of ginkgolides A using external calibration method by UHPLC-ELSD and FI-HRMS in Finzelberg Extrakte–5 extract.

Result obtained by UHPLC-ELSD assay: 0.85 ± 0.07

Result obtained by FI-HRMS assay: 1.11 ± 0.01

b. Calculation.

$$S = \sqrt{\frac{(3-1)0.07^2 + (3-1)0.01^2}{3+3-2}}$$

$$S = 0.050$$

$$t = \frac{(1.11 - 0.85)}{0.050\sqrt{\left(\frac{1}{3} + \frac{1}{3}\right)}}$$

$$t = 6.37$$

c. *t*-table

Table A.2 The *t*-distribution

Value of *t* for a confidence interval of Critical value of |t| for *P* values of number of degrees of freedom	90% 0.10	95% 0.05	98% 0.02	99% 0.01
1	6.31	12.71	31.82	63.66
2	2.92	4.30	6.96	9.92
3	2.35	3.18	4.54	5.84
4	2.13	2.78	3.75	4.60
5	2.02	2.57	3.36	4.03
6	1.94	2.45	3.14	3.71
7	1.89	2.36	3.00	3.50
8	1.86	2.31	2.90	3.36
9	1.83	2.26	2.82	3.25
10	1.81	2.23	2.76	3.17
12	1.78	2.18	2.68	3.05
14	1.76	2.14	2.62	2.98
16	1.75	2.12	2.58	2.92
18	1.73	2.10	2.55	2.88
20	1.72	2.09	2.53	2.85
30	1.70	2.04	2.46	2.75
50	1.68	2.01	2.40	2.68
∞	1.64	1.96	2.33	2.58