



RESEARCH ARTICLE

SIMULTANEOUS QUALITATIVE AND QUANTITATIVE ANALYSIS OF CERTAIN FLAVONOID GLYCOSIDES AND TERPENE LACTONES IN PHARMACEUTICAL PRODUCTS CONTAINING *GINKGO BILOBA L.* LEAF EXTRACT BY LC-TOF/MS

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ABSTRACT

In this study, three drugs and three dietary supplements containing *G. biloba* leaf extract were obtained from pharmacies and two dietary supplements were obtained from herbalists. Certain flavonoid glycosides and ginkgolide A, B, C, and J amounts of these products were determined simultaneously using LC-TOF/MS. The total phenol and flavonoid content of these products were also determined with spectrophotometric technique.

Objective: We aimed to emphasize that adulteration is still a critical problem in herbal products frequently used during the Covid-19 pandemic and propose a fast, validated analytical method for detecting adulteration.

Conclusions: The qualitative and quantitative results of all drugs and dietary supplements obtained from the pharmacy were compatible with the information declared by the manufacturer. However, neither qualitative nor quantitative flavonoid glycosides or ginkgolides A, B, and C were detected, although the total phenolic and flavonoid values were very high in one of the dietary supplements taken by the herbalist. The study's data is considered significant, mainly due to the considerable increase in interest in herbal products during the Covid-19 pandemic.

Keywords: *G. Biloba, Drug, Dietary Supplement, Adulteration, Validation, LC-TOF/MS.*

1. INTRODUCTION

G. biloba is one of the most widely used natural medicinal plants, containing many bioactive components with therapeutic efficacy. Environmental factors have little effect on the survival of *G. biloba*, the only surviving species of the Ginkgoaceae family [1]. In fact, after the explosion of the atomic bomb in Hiroshima, Japan in 1946, the first plant to germinate was *G. biloba* [2]. *G. biloba*, a

tree native to China and used as a traditional medicinal plant for over 2,000 years [3] is now grown in Europe, Asia, Argentina, North America, and New Zealand [4].

With the discovery of the effectiveness of *G. biloba* leaf extract in peripheral blood circulation disorders and cerebral vascular diseases in the 1960s [5-8] it began to be used for different purposes. Pharmaceuticals containing *G. biloba* standardized leaf extract (EGb 761) are used in the treatment of tinnitus [9], cognitive impairment and Alzheimer's disease [10], retinal diseases [11], cardiovascular disease [12], cerebrovascular ischemia [13], peripheral vascular disease [14] and diabetic nephropathy [15]. Standardized *G. biloba* leaf extract is one of the most widely used herbal products and/or dietary supplements in the world [16-19] and it is possible to find different forms of pharmaceutical products such as tablets, capsules, solutions, etc. containing *G. biloba* approved by the Ministry of Health in Turkey [20].

Although there are many different bioactive compounds such as terpene lactones, flavonoids, fatty acids, proanthocyanidins, and polysaccharides in *G. biloba* leaf extract, [21,22] flavonoids and terpene lactones are considered to be the two main groups of biologically effective components [23-26]. Some studies performed on standardized *G. biloba* leaf extract show that it contains approximately 24% flavonol glycoside (22%- 27%) and 6% terpene lactone (2.6% - 3.2% bilobalide, 2.8% - 3.4% ginkgolide A (GA), ginkgolide B (GB), ginkgolide C (GC), and ginkgolide J (GJ)) [27-30]. Therefore, qualitative, and quantitative determination of flavonol glycosides and terpene lactones is one of the important parameters for evaluating the quality of products containing *G. biloba* [31]. *G. biloba* leaf extract contains more than 30 flavonol glycosides [16,25,29]. These flavonol glycosides are converted to three aglycones (Quercetin (QUE), Kaempferol (KAE), and Isorhamnetin (ISH)) by hydrolytic reactions. Therefore, the total amount of flavonoid aglycones is multiplied by 2.51 to calculate the total amount of flavonoid glycosides [16].

The mentioned values should be stated on the labels of products using standardized EGb 761 extract. However, studies carried out on the products containing *G. biloba* leaf extract in the world market reported varying concentrations of the contents [30, 31]. This situation reveals that the active ingredient can be mixed with cheaper and easily available botanical materials [32]. Unfortunately, due to some economic concerns, adulteration is still encountered in these products. This situation is a source of great danger to product reliability [33,34]. In the determination of adulteration, chromatographic and spectroscopic analytical methods are used to detect and characterize bioactive components. The most widely used techniques are thin-layer chromatography (TLC), inductively coupled plasma mass spectrometry (ICP-MS), high-performance liquid chromatography (HPLC), gas chromatography (GC), nuclear magnetic resonance spectroscopy (NMR), and near-infrared spectroscopy (NIR) [25,32,35-38]. In addition, tandem systems such as liquid chromatography/time-of-flight/mass spectrometry (LC-TOF/MS), which we used in our study, are an option.

In addition to the fact that pharmaceutical products prepared from *G. biloba* leaf extract and standardized extract have many useful applications, the efficacy and content controls of dietary supplements containing these herbal products and mainly supplied out of pharmacies should be evaluated scientifically. This is especially important when considering the frequency of people using herbal products due to the Covid 19 pandemic, which has been affecting the world for the last three

years. Because anti-inflammatory and antioxidant agents have been claimed to be crucial in reducing COVID-19 patients' problems in the research [39].

For this purpose, it is aimed to control the content of selected pharmaceuticals and dietary supplements containing *G. biloba* leaf extract in the Turkish market with a fast, easy-to-apply and validated analytical method. In addition, the total phenol content of these products was determined using the Folin-Ciocalteu method, and the flavonoid content was determined using the aluminum chloride method. Thus, both content comparisons and phenolic capacities of pharmaceutical preparations containing *G. biloba* leaf extract were evaluated to determine possible adulteration.

2. MATERIAL and METHODS

2.1. Chemicals and Instruments

QUE, KAE, GA, GB, and GC were purchased from Sigma Aldrich (St. Louis, MO, USA). HPLC purity formic acid and methanol were provided from Merck (Darmstadt, Germany). Sodium carbonate, sodium nitrite, Folin reagent, gallic acid, and aluminum chloride were purchased from Sigma Aldrich (St. Louis, MO, USA). UV-1601 spectrophotometer (UV-1601, Shimadzu, Japan) and LC-TOF/MS (6545 Accurate-Mass, Agilent, USA) were used throughout this work.

2.2. Preparation of Samples and Standards

Stock solutions of QUE, KAE, GA, GB, and GC, used as standards in the analyses, were prepared with methanol. The extraction of drugs (D, E, F), dietary supplements (A, B, C, G, H) used in the study (Table 1) was carried out as described in sections 2.3 and 2.4. Before LC-TOF/MS analysis, all standards and samples were filtered through 0.45 µm membrane filters (Millipore).

Table 1. Information on drugs and dietary supplements.

Product Code	Form	Formulation	Standardized amount of extract	Purchased from
A	Dietary supplement	Capsule	<i>G. biloba</i> leaf extract 90 mg	Pharmacy
B	Dietary supplement	Capsule	<i>G. biloba</i> leaf extract 100 mg	Pharmacy
C	Dietary supplement	Capsule	<i>G. biloba</i> leaf extract 125 mg	Pharmacy
D	Drug	Tablet	<i>G. biloba</i> leaf extract 120 mg	Pharmacy
E	Drug	Tablet	<i>G. biloba</i> leaf extract 120 mg	Pharmacy
F	Drug	Tablet	<i>G. biloba</i> leaf extract 80 mg	Pharmacy

G	Dietary supplement	Tablet	<i>G. biloba</i> leaf extract 60 mg	Herbalist
H	Dietary supplement	Powder	<i>G. biloba</i> leaf extract 100 mg	Herbalist

2.3. Acid Hydrolysis Extraction for Flavonoid Glycosides

Extraction was performed with some modifications to the method proposed by Czige et al. [40] Ten tablets and 10 capsules were crushed separately in mortars and homogenized. An amount equivalent to 1 tablet or capsule was weighed and taken from the resulting homogeneous mixture. 45 mL of methanol (99%, Merck) was added to the weighed samples, and they were extracted ultrasonically. Then, 5 mL concentrated HCl (37%, Merck) and 9 mL ultrapure water were added. They were refluxed in the oil bath at 100-135 °C in the condenser. The obtained pink-red extracts were stored in the refrigerator at +4 °C for LC-TOF/MS analysis. The same procedures were repeated by taking 100 mg from the powder samples obtained from the herbalist.

2.4. Liquid-Liquid Extraction with Ethyl Acetate for Terpene Lactones

Extraction was performed with some modifications to the method proposed by Li et al. [28] Ten tablets and 10 capsules were crushed separately in mortars and homogenized. An amount equivalent to 1 tablet or capsule was weighed and taken from the resulting homogeneous mixture. 45 mL ultrapure water was added to the weighed samples and their extraction was done ultrasonically. After each extraction, the dissolved fractions were collected and filled to a final volume of 50 mL with distilled water. The extracts were taken into a separating funnel and liquid extraction was achieved three times with 20 mL of ethyl acetate. The ethyl acetate organic phases were combined and evaporated to dryness. The resulting residue was diluted with 10 mL analytical grade (99%) MeOH. It was stored in the refrigerator at +4 °C for LC-TOF/MS analysis. The same procedures were repeated by taking 100 mg of the powder samples obtained from the herbalist.

2.5. Equipment and Chromatographic Conditions

Qualitative and quantitative analysis of samples was performed with an Agilent LC-TOF/MS. The equipment and chromatographic are shown in Table 2.

Table 2. LC-TOF/MS conditions for analysis.

Equipment	Parameter	Conditions
LC-TOF/MS	Column	Agilent Poroshell 120 EC-C18 (3.0×50 mm, particle size 2.7 µm)
	Mobile phase	Gradient elutions of 0.1% formic acid (A) and 99% methanol (B) solutions 0 to 2 min, 0%B→5%B; 2 to 4 min, 5%B→95%B, 4 to 30 min, 95%B
	Analysis time	32 min
	Column temperature	30 °C
	Injection volume	10.0 µL
	Mobile phase	1.0 mL/min

flow rate	
MS system and ion source	Agilent 6545 Accurate-Mass TOF/MS Negative ion mode Drying gas flow: 10.0 L/min, Nebulizer pressure: 35 psi Gas drying temperature: 325 °C Sheath gas temperature: 400 °C Sheath gas flow: nitrogen at 12 L/min Scan range: m/z 100 to 1400

2.6. Validation Parameters

Limit of Detection (LOD), Limit of Quantification (LOQ), measurement range and linearity, accuracy, and precision parameters were examined for the validation of the proposed method. In addition, the precision of the proposed method was evaluated intraday and between days. Recovery calculations were carried out to determine the accuracy of the proposed method. Therefore, LC-MS analyses were done by performing the extractions of the standards at known concentrations as in sections 2.3 and 2.4. The recovery values obtained were calculated using Eq. 1 [35].

$$\text{Recovery (\%)} = (\text{amount found} - \text{original amount}) / \text{amount added} \times 100\% \quad (1)$$

2.7. Total Phenol Content

The total phenol content of the samples was determined spectrophotometrically using the Folin-Ciocalteu method [41]. First, 100 µL of each sample (A, B, C, D, E, F, G, and H) was prepared as in Section 2.3, then 7900 mL distilled water and 500 µL Folin-Ciocalteu reagent were added (10%, v/v in water). This mixture was vortexed and incubated for 2 min at room temperature in the dark. Immediately after, 1.5 mL 20% (w/v) Na₂CO₃ solution was added to the mixture and vortexed again. The resulting mixture was kept in the dark for 2 h at room temperature, and the absorbance of the blue solution formed was measured in a UV-Vis spectrophotometer at a wavelength of 765 nm against the blank. Gallic acid was used as standard. The results obtained were calculated as the mean of three measurements and expressed mg GAE/g.

2.8. Total Flavonoid Content

The total flavonoid amounts of the samples were measured spectrophotometrically according to the aluminum chloride/sodium nitrite method [42]. First, 500 µL of each sample (A, B, C, D, E, F, G, and H), was prepared as in section 2.3. It was transferred to a tube containing 3 mL distilled water and vortexed after adding 0.3 mL 5% aqueous NaNO₂ solution. After 5 min, 0.3 mL 10% aqueous AlCl₃ solution was added to the mixture. After 1 min, 2 mL 1 M NaOH solution was added to the mixture, and the tube was filled to a total volume of 10 mL with distilled water. The final mixture was vortexed again and the absorbance was measured at 510 nm against water. The results obtained were calculated as the mean of three measurements and expressed mg Quercetin/g.

3. RESULTS and DISCUSSION

3.1. Method Validation

Calibration lines with acceptable linearity were obtained for QUE, KAE, GA, GB, and GC. Correlation coefficients were obtained with $r^2 > 0.98$. LOD, LOQ, precision and recovery values were calculated as described in Section 2.6. The data obtained as a result of the validation studies were summarized in Table 3.

Table 3. Validation parameters of QUE, KAE, GA, GB, and GC.

	Calibration curve	R ²	Linearity mg/L	LOD mg/L	LOQ mg/L	Repeatability		Recovery %
						Intra Day RSD%	Between Days RSD%	
QUE	Y=4E+06x+2E+07	0.988	0.625-5	0.256	0.853	1.91	3.01	98.72 ± 0.04
KAE	Y=6E+06x+9E+06	0.998	0.625-10	0.391	1.304	1.95	3.90	98.25 ± 0.09
GA	Y=145657x+343882	0.995	2.5-20	0.263	0.878	1.72	2.64	97.32 ± 0.34
GB	Y=2E+06x+3E+06	0.990	1.25-10	0.086	0.287	1.13	2.44	96.33 ± 0.13
GC	Y=2E+06x+2E+06	0.992	1.25-10	0.136	0.453	1.31	2.40	96.84 ± 0.21

3.2. Retention Times of Standards and Samples for LC-TOF/MS Analysis

The analyses were performed under the conditions specified in Section 2.5 for the individually prepared solutions of all analyzed standard substances (QUE, KAE, GA, GB, and GC). Retention times were determined for each standard. Meanwhile, the analysis of the mixture containing all the standards was also performed. The chromatogram obtained for the standards was shown in Figure 1, and the retention time and molecular ion peaks of each standard were shown in Table 4.

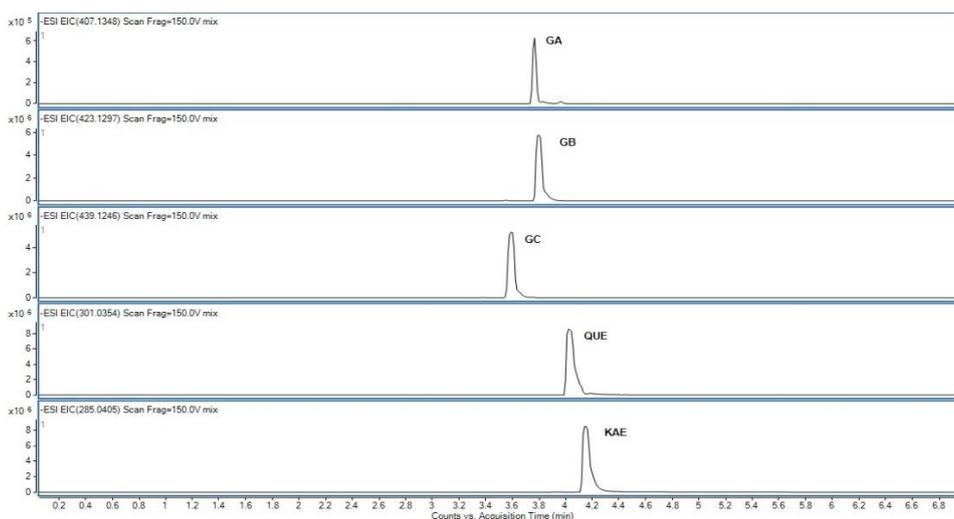


Figure 1. Chromatogram of standards.

Table 4. Molecular ion peaks and retention times of the standards.

Standards	M^-	t_R (min)
QUE	301.0379	4.03
KAE	285.0433	4.15
GA	443.1157	3.77
GB	423.1331	3.80
GC	439.1283	3.60

On the other hand, it is seen that there are studies carried out using HPLC-UV in the literature [43,44]. However, the reason for using LC-TOF/MS in this study is to avoid peak overlaps [45] since terpene lactones have low ϵ -values for maximum absorbance at about 219 nm. The chromatogram obtained for the samples was shown in Appendix A. Retention times of QUE, KAE, GA, GB, and GC present in the analyzed pharmaceutical samples were given in Table 5. The retention times of QUE, KAE, GA, GB, and GC in the samples were found to be compatible with the retention times of the standards. QUE, KAE, GA, GB, and GC were not found in the H sample obtained from herbalists.

Table 5. Retention times of the samples.

Samples	t_R (min)				
	QUE	KAE	GA	GB	GC
A	4.03	4.15	3.77	3.80	3.60
B	4.03	4.15	3.77	3.81	3.59

C	4.03	4.15	3.76	3.81	3.59
D	4.03	4.15	3.77	3.81	3.59
E	4.04	4.15	3.77	3.81	3.59
F	4.03	4.14	3.76	3.80	3.59
G	4.03	4.14	3.76	3.80	3.60
H	-	-	-	-	-

3.3. Quantification of Total Flavonoids and GA, GB, GC, and GJ

The results of analyses showed that all the drugs and dietary supplements obtained from the pharmacy qualitatively contained QUE, KAE, GA, GB, and GC. The standard curve of GB for GJ and the calibration curve of QUE for ISH were used due to their similar structures and close molecular weights. The amounts of GJ and ISH were calculated using the molecular weight correction factor. As previously stated, this approach could be used [46]. The total flavonoid value was calculated by multiplying the amounts of QUE, BAE, and ISH with the appropriate conversion factors, and the results were compared with the declared values. The amount per tablet/capsule in all drugs and dietary supplements was detected within the declared limits. However, neither qualitative nor quantitative QUE, KAE, GA, GB or GC were present in the dietary supplement (H) obtained from the herbalist. The amounts found in the other dietary supplement (G) were detected as much lower than stated on the label (Table 6, 7).

Table 6. Total flavonoid glycosides content of 8 samples containing *G. biloba* leaf extracts (n=3, Mean ± SD).

Sample	QUE (mg/tb or capsule)	KAE (mg/tb or capsule)	ISH (mg/tb or capsule)	Total (mg)	Total Flavonoid Glycosides Found (mg)	Total Flavonoid Glycosides Expected (mg)
A	3.98 ± 0.02	3.59 ± 0.03	1.11 ± 0.02	8.68	21.79	19.8-24.30
B	4.36 ± 0.03	4.05 ± 0.04	1.35 ± 0.03	9.76	24.50	22-27
C	5.17 ± 0.01	5.11 ± 0.03	1.85 ± 0.04	12.13	30.44	27.5-33.75
D	5.02 ± 0.04	4.78 ± 0.05	1.58 ± 0.06	11.38	28.56	26.4-32.40
E	4.90 ± 0.06	4.79 ± 0.04	1.51 ± 0.05	11.20	28.11	26.4-32.40
F	3.11 ± 0.03	3.34 ± 0.02	0.93 ± 0.04	7.38	18.52	17.6-21.60
G	1.86 ± 0.02	1.52 ± 0.05	0.25 ± 0.03	3.63	9.11	13.2-16.20
H	-	-	-	-	-	22-27

Table 7. GA, GB, GC, and GJ content of samples containing *G. biloba* leaf extracts (n=3, Mean ± SD).

Sample	GA (mg/tb or capsule)	GB (mg/tb or capsule)	GC (mg/tb or capsule)	GJ (mg/tb or capsule)	GA GB GC GJ Found (mg)	GA GB GC GJ Expected (mg)
A	0.90 ± 0.02	0.88 ± 0.03	0.62 ± 0.04	0.13 ± 0.02	2.53	2.52-3.06
B	1.04 ± 0.04	0.97 ± 0.05	0.63 ± 0.02	0.21 ± 0.03	2.85	2.8-3.40
C	1.54 ± 0.03	1.07 ± 0.02	0.71 ± 0.04	0.47 ± 0.03	3.79	3.5-4.25
D	1.46 ± 0.05	0.95 ± 0.04	0.69 ± 0.02	0.46 ± 0.03	3.56	3.36-4.08
E	1.38 ± 0.02	0.90 ± 0.03	0.67 ± 0.05	0.47 ± 0.04	3.42	3.36-4.08
F	0.52 ± 0.06	0.30 ± 0.02	0.22 ± 0.04	0.05 ± 0.03	1.09	2.24-2.72
G	0.31 ± 0.04	0.25 ± 0.05	0.20 ± 0.03	0.05 ± 0.02	0.81	1.68-2.04
H	-	-	-	-	-	2.8-3.40

The results obtained from our study show that adulteration is still a very serious problem, especially in dietary supplements purchased out of pharmacies. In our study, QUE, KAE, GA, GB, and GC could not be determined qualitatively and quantitatively in H sample containing *G. biloba* obtained from herbalists. In addition, there are differences between the reported values and the found values in the G sample obtained from the herbalist. This situation is similar to other studies reported in the literature [47,48].

3.4. Total Phenol and Flavonoid Analysis

The data summarized in Table 8 showed that, as expected, the highest total phenol, and flavonoid values were found in the sample containing the highest *G. biloba* standard leaf extract (C). On the other hand, the total phenol and flavonoid values determined in the tablet sample obtained from herbalists (G) were at the lowest level compared to the other tablets. Although no bioactive compounds in the standardized *G. biloba* extract was qualitatively detected in the powder sample taken from the herbalist, total phenol, and flavonoid values were at the highest level. This situation suggests that there may be adulteration in the sample.

Table 8. Total phenolic and flavonoid values for the samples (n=3, Mean ± SD).

Sample	Total Phenol mg (GAE)/g	Total Flavonoid mg (KUE)/g
A	35.51 ± 0.52	16.03 ± 0.29
B	36.51 ± 0.37	18.2 ± 0.50
C	40.48 ± 0.11	27.53 ± 0.29
D	38.14 ± 0.14	23.7 ± 0.51
E	37.47 ± 0.10	22.87 ± 0.28

F	32.78 ± 0.10	12.37 ± 0.30
G	18.42 ± 0.15	9.03 ± 0.55
H	50.05 ± 0.52	45.37 ± 0.31

4. CONCLUSION

In this study, qualitative and quantitative analysis of drugs and dietary supplements containing *G. biloba* leaf extract available in the Turkish market was performed with a fast, easy, and validated analytical method. The highest total phenol and flavonoid values among the products purchased from the pharmacy were in the tablet containing the highest amount of *G. biloba* leaf extract. The total phenol and flavonoid values for the tablet sample purchased from the herbalist were the lowest. On the contrary, the total phenol and flavonoid values of the powder sample obtained from the herbalist were the highest compared to other pharmaceutical products. In this case, the most surprising point according to the LC-TOF/MS results was that the flavonoid glycosides and ginkgolide A, B, and C found in *G. biloba* were not detected in the powder sample taken from the herbalist. The fact that the dietary supplements showed such high total phenol and flavonoid values while none of the bioactive components of *G. biloba* exist in its content indicates that it may have been adulterated with other botanical materials with lower costs.

Table 9 compares the findings of this study to those of other *G. biloba* studies in the literature. The findings indicate that adulteration is still a significant issue in various *G. biloba* samples.

Table 9. Comparison of the results of the present study and other studies on the analysis of *G. biloba*.

Sample	Method	Finding	Reference
<i>G. biloba</i> food supplements	HPLC- UV LC- MS/MS	Adulteration was determined through analyses performed on selected samples.	40
<i>G. biloba</i> L. phytopharmaceuticals	HPLC- UV	The proposed methods were used successfully to determine terpenes and flavonoids in four phytopharmaceutical preparations from the Egyptian market.	44
<i>G. biloba</i> L. pharmaceuticals	HPLC-DAD LC-MS	The study revealed that food supplements were mixed with rutin to reach the expected amount of flavonoid glycosides.	49
<i>G. biloba</i> solid oral dosage form	HPLC-PDA	According to the findings, appropriate quality control measures should be implemented to ensure the quality, safety, and efficacy of commercially available <i>G. biloba</i> products.	50

<i>G. biloba L.</i> drugs and three dietary supplements	LC-TOF/MS	Serious adulteration was noticed in dietary supplements containing <i>G. biloba</i> that were not purchased from a pharmacy.	This study
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The study results reveal that we should be more cautious about the quality of these products, especially given the frequent use of herbal products during the Covid 19 pandemic.

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APPENDIX A

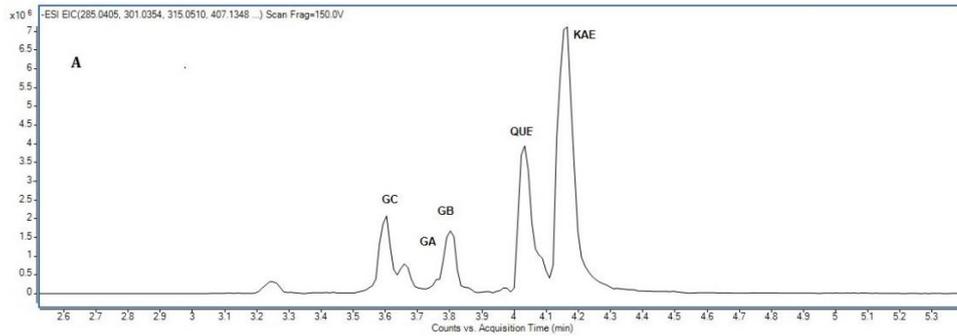


Figure A1. Chromatogram of A (dietary supplement) sample obtained from pharmacy.

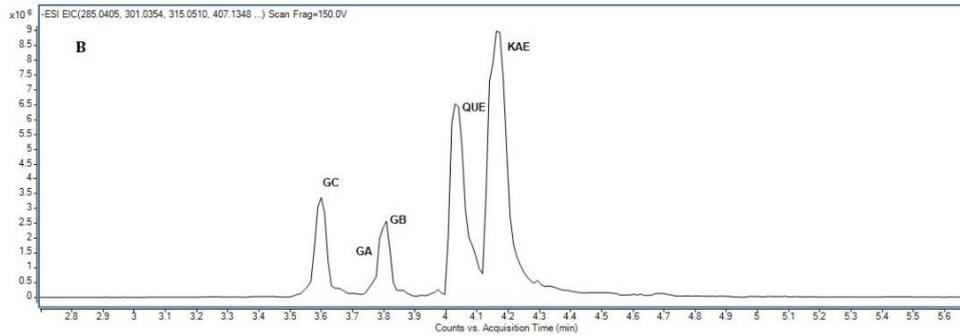


Figure A2. Chromatogram of B (dietary supplement) sample obtained from pharmacy.

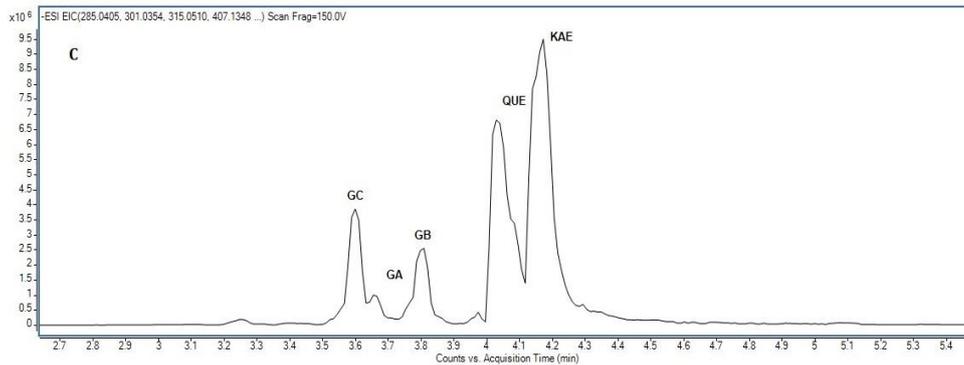


Figure A3. Chromatogram of C (dietary supplement) sample obtained from pharmacy.

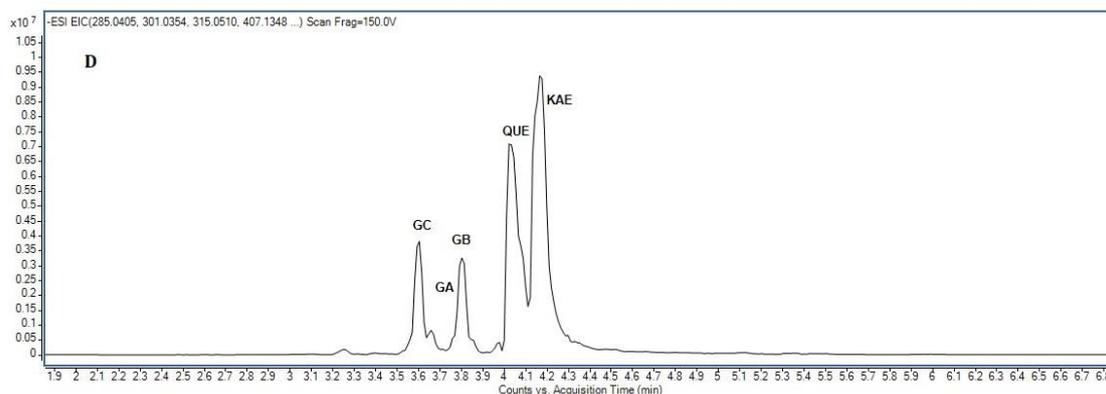


Figure A4. Chromatogram of D (drug) sample obtained from pharmacy.

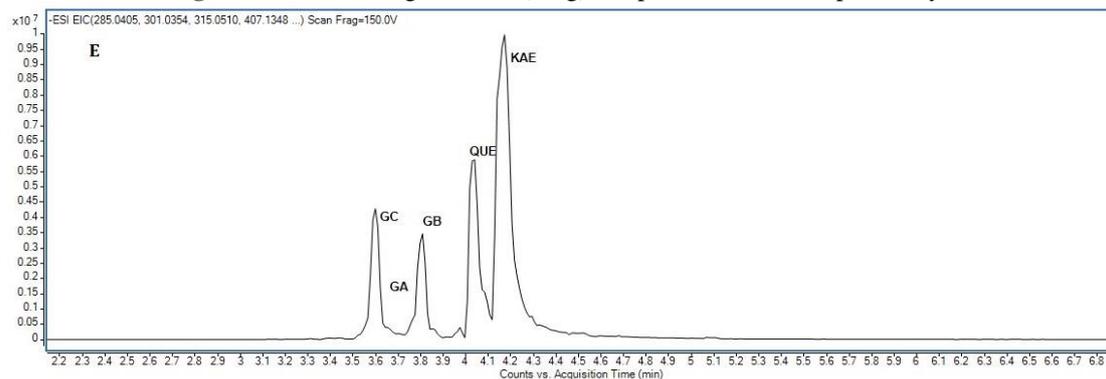


Figure A5. Chromatogram of E (drug) sample obtained from pharmacy.

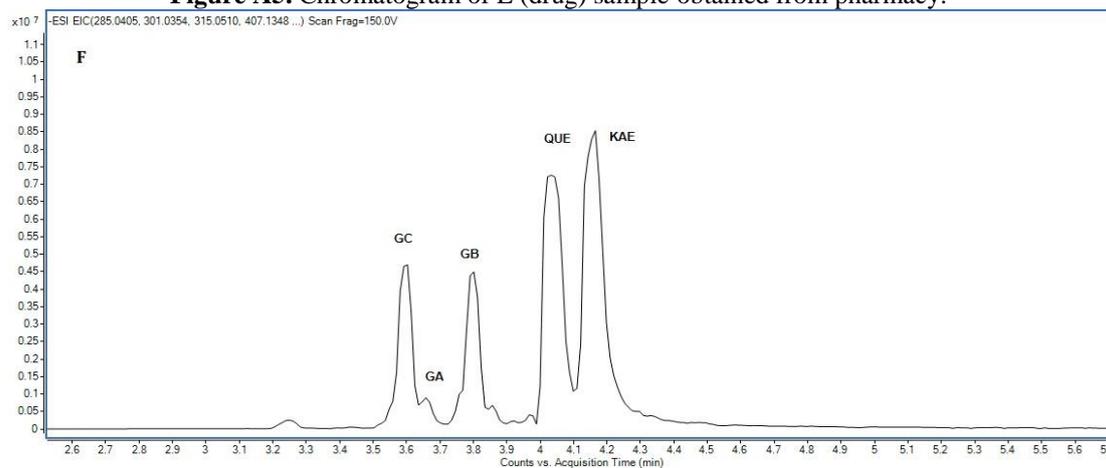


Figure A6. Chromatogram of F (drug) sample obtained from pharmacy.

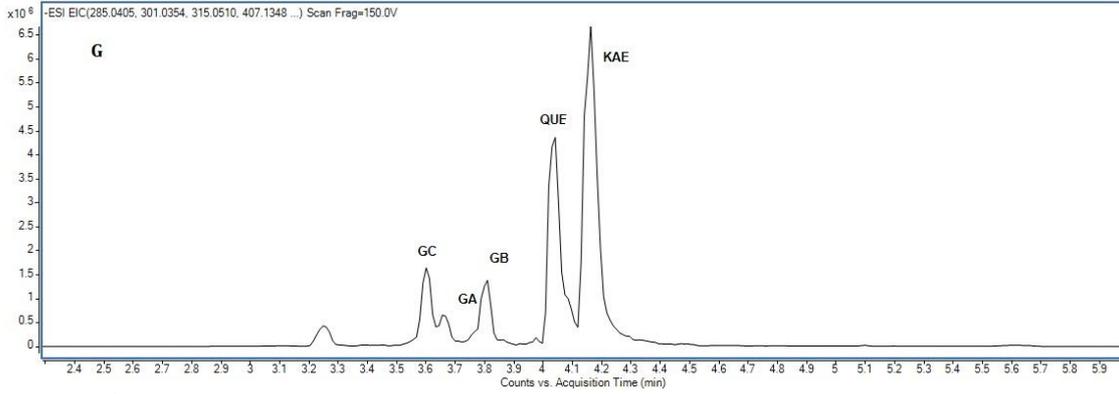


Figure A7. Chromatogram of G (dietary supplement) sample obtained from herbalist.

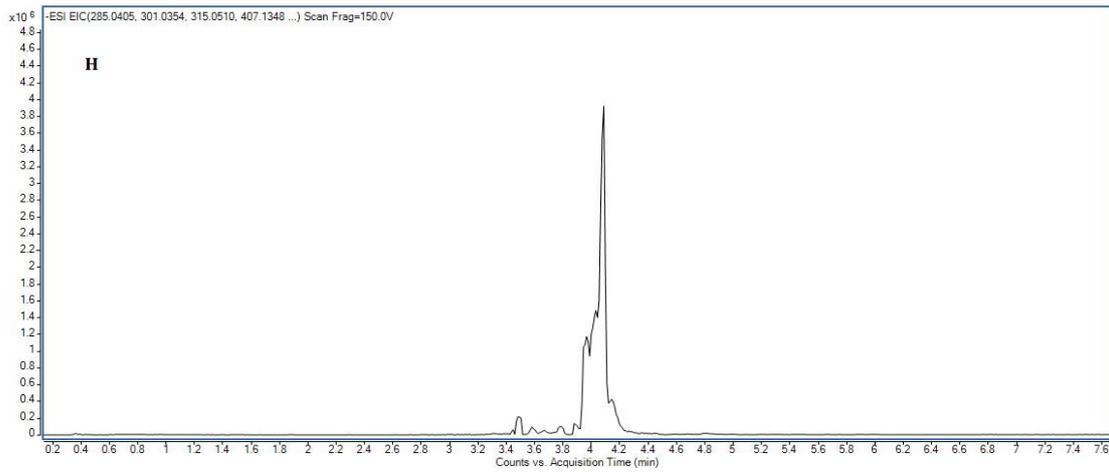


Figure A8. Chromatogram of H (dietary supplement) sample obtained from herbalist.