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Journal of Pharmaceutical and Biomedical Analysis 37 (2005) 723–731
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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The simultaneous determination of selected flavonol glycosides and aglycones in *Ginkgo biloba* oral dosage forms by high-performance liquid chromatography–electrospray ionisation–mass spectrometry

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Received 27 September 2004; received in revised form 22 November 2004; accepted 22 November 2004
Available online 7 January 2005

Abstract

An accurate, precise and sensitive liquid chromatography-tandem mass spectrometric (LC–MS–MS) method was developed for the determination of two flavonol glycosides, rutin and quercitrin, together with the algycone markers, quercetin, kaempferol and isorhamnetin in several *Ginkgo biloba* solid oral dosage forms. In addition, a novel quercetin glycoside, not yet reported in Ginkgo extracts, was identified. Liquid chromatography was performed using a minibore high-performance liquid chromatography (HPLC) column (150 mm × 2.0 mm i.d.) and a one step gradient of acetonitrile–formic acid (0.3%) at a flow rate of 0.5 ml/min. Baseline separation of the five selected flavonol marker compounds was achieved within 20 min at 45 °C. Tandem mass spectrometry was performed using electrospray ionisation (ESI) in the negative ion mode. The marker compounds exhibited linearity over the range of 3–26 μg/ml and intra- and inter-day standard deviations were better than 7% and 16%, respectively. All Ginkgo products investigated were found to contain varying amounts of target analytes.

Keywords: *Ginkgo biloba*; Flavonols; LC–MS–MS; Validation

1. Introduction

Increasing sales of herbal products indicate a worldwide concurrent surge of natural product use [1]. Due to the increasing popularity of phytomedicines, concerns regarding the quality, safety and efficacy of these preparations have come to the fore. Herbal products are currently classified as dietary supplements in different countries, and are generally not required to undergo the usual quality control tests as expected for allopathic medicines [2]. Research has indicated that the content of active principle(s) in natural products may vary depending on a number of factors such as the season and time of harvesting, growing conditions, plant genetics, harvesting and processing techniques [3,4]. In addition, adulterants and contaminants, as well as the presence of degradation products, are not uncommon [1]. Incidences of adverse effects [5], particularly interactions with orthodox medicines [1,6], have indicated that stringent quality control procedures need to be implemented.

Ginkgo biloba is an ancient Chinese phytomedicine used to treat various ailments including circulatory and demential disorders. Standardized leaf extracts have been suggested to be clinically effective in the treatment of Alzheimer's disease, depression, diabetic neuropathy, impotency, memory impairment, peripheral vascular disease, intermittent claudication, vertigo and tinnitus [7]. The positive effects of Ginkgo extracts are thought to result from the synergistic action of two distinct groups of compounds, the flavonoids and triterpene lactones [8]. The flavonoids are responsible for the free radical scavenging effects of Ginkgo [9–11], while the

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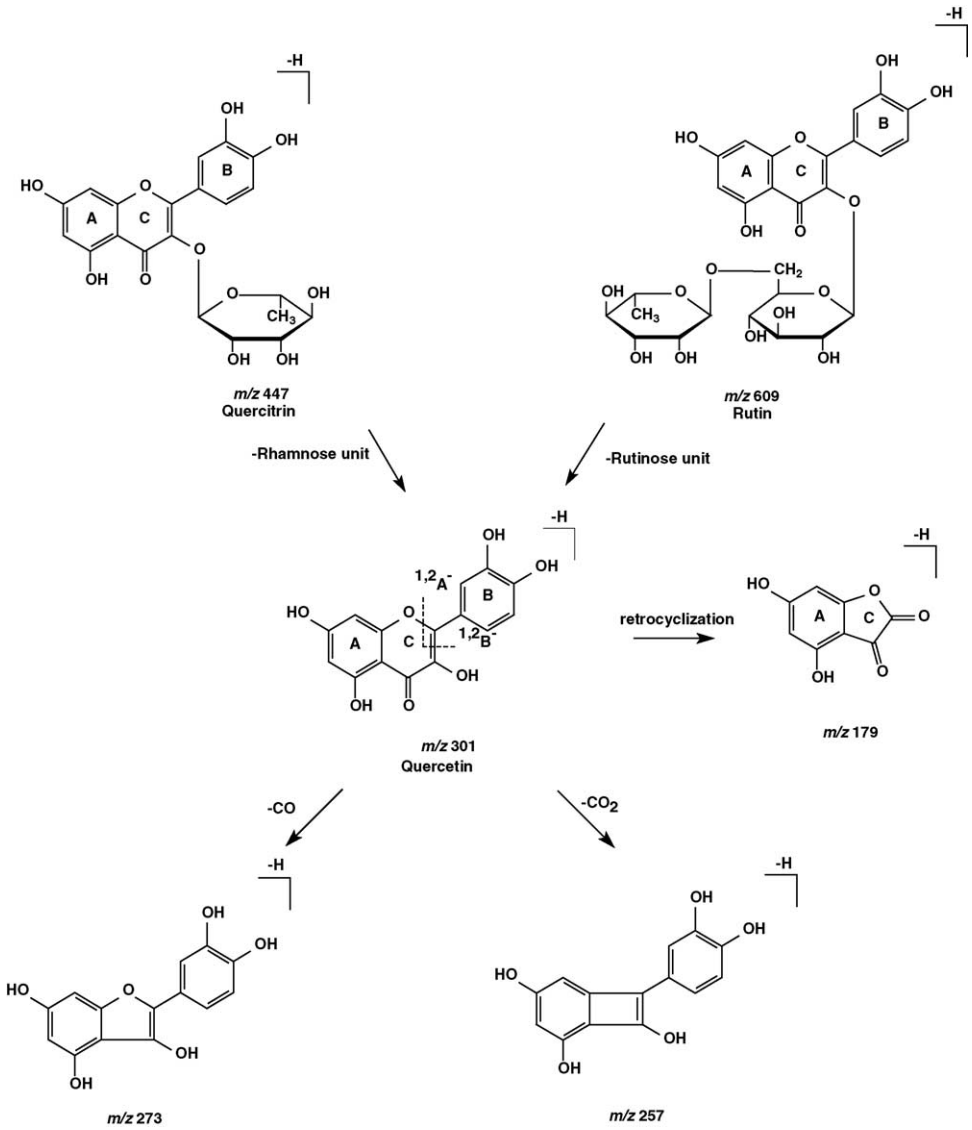
doi:10.1016/j.jpba.2004.11.052

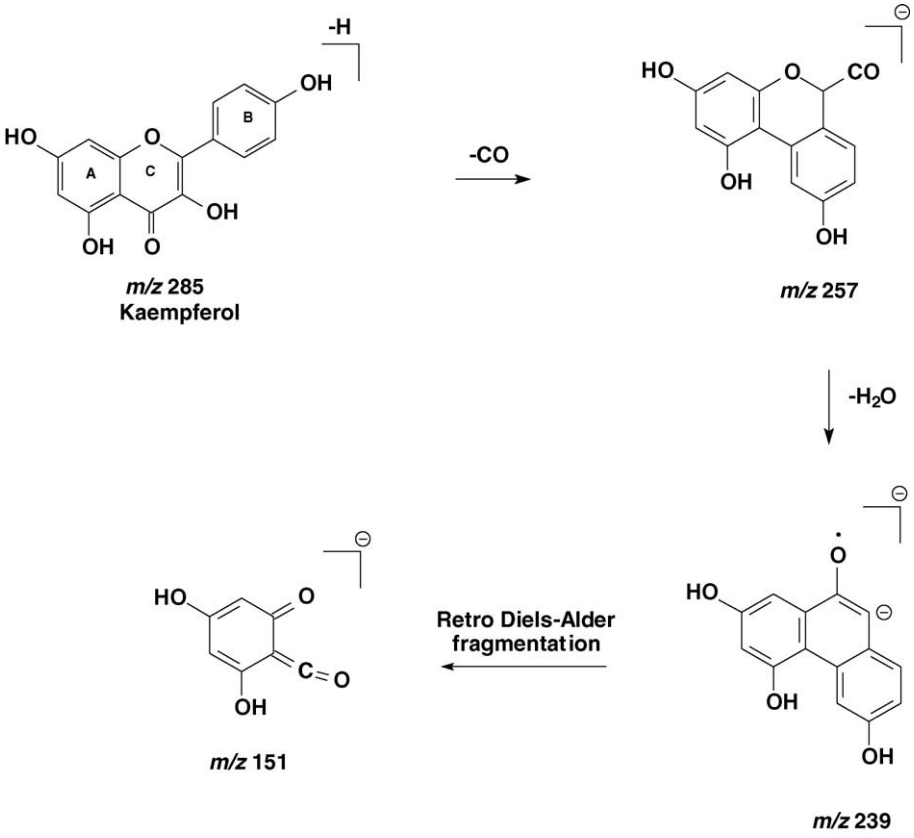


Scheme 1. Fragmentation patterns of rutin, quercitrin and quercetin.

ginkgolides are potent anti-platelet factor (PAF) antagonists [12]. Attempts to standardize *Ginkgo biloba* products have employed various analytical techniques using the flavonoids and/or ginkgolides as marker compounds.

The flavonoids are a large group of polyphenols which occur naturally in the plant kingdom [13,14] and include flavone and flavonol glycosides, acylated flavonol glycosides, biflavonoids, flavane-3-ols and proanthocyanidins [15]. Of these, special attention has been given to the flavonols due to their abundance in *Ginkgo biloba* extracts. Numerous flavonol glycosides have been identified [16] as derivatives of the flavonol aglycones, quercetin, kaempferol and isorhamnetin (Schemes 1–3). The current approach to standardization of flavonols in Ginkgo extracts is by calculation of the total flavonol glycoside content from the aglycone concentration in extracts after acid hydrolysis. Although this procedure is relatively simple and widely accepted, the aglycones already present in extracts and calculations based on the average glycoside mass result in exaggerated reported flavonol glycoside content [17]. The presence of glycosides, which may hydrolyse during extraction and/or incorrect storage are therefore useful quality control indicators. An increase in the ratio of aglycones to glycosides in extracts signifies degradation [18]. It has been suggested that gastrointestinal absorption of quercetin in its glycosidic form is better than that of the aglycone form [19]. Although reference standards for all flavonol glycosides are not available, relevant flavonol glycoside markers can be chosen for analytical techniques to ensure comprehensive standardization.

The flavonols have strong chromophores resulting in reversed-phase high-performance liquid chromatography (HPLC) with UV detection being the most common method of analysis [20]. On the other hand, liquid chromatography (LC) coupled to mass spectrometry (MS) has been demon-

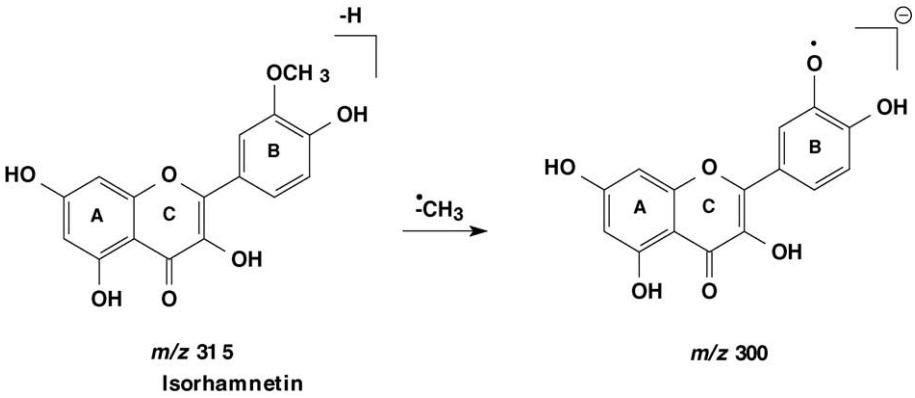


Scheme 2. Proposed fragmentation pattern of kaempferol [25].

strated to be a powerful tool for the identification of natural products in crude plant extracts owing to their soft ionization which favours the analysis of flavonols, in addition to its high sensitivity and specificity [21,22]. Tandem mass spectrometry has the additional advantage of ensuring unequivocal identification and quantification of target analytes in a tablet matrix as opposed to UV detection where identification is primarily by retention time of the marker compound. To date, only a direct infusion, qualitative MS method has been developed to identify flavonol glycosides in standardized *Ginkgo biloba* extracts [17].

This paper describes a LC–MS–MS method to quantify five useful flavonol marker compounds in *Ginkgo biloba* dosage forms. In addition to the usual aglycone markers, quercetin, kaempferol and isorhamnetin, two flavonol glycosides, rutin and quercitrin were included as useful compounds to assist in evaluating the quality of several Ginkgo dosage forms. Furthermore, the method allows another quercetin glycoside not yet reported in *Ginkgo biloba* extracts, to be identified.

This method has the necessary accuracy, reproducibility, precision and sensitivity to quantitatively determine trace



Scheme 3. Fragmentation pattern of isorhamnetin.

amounts of the relevant markers with minimal sample clean-up.

2. Experimental

2.1. Reagents and chemicals

Formic acid (analytical grade) was purchased from Merck (Darmstadt, Germany) and acetonitrile (HPLC grade) was obtained from BDH chemicals (Poole, UK). Rutin and quercetin were purchased from Sigma (St. Louis, MO, USA) and quercitrin, kaempferol and isorhamnetin from Indofine Chemical Company (New Jersey, USA). Water was purified in a Milli-Q system (Millipore, Bedford, MA, USA) and low protein binding durapore (PVDF) filters were purchased from the same source. Six *Ginkgo biloba* products (I, II, III, IV, V and VI) were purchased from a local pharmacy in Grahamstown, South Africa. Four of the products were solid oral dosage forms and two preparations contained herbal extract in a gelatin capsule.

2.2. Standards and sample solutions

Separate stock solutions of the reference standards were made in methanol–water (50:50). A working solution of the combined standards was subsequently prepared in methanol–water (50:50) and diluted to provide a series of analytical standards ranging from 3 to 26 μg/ml for use in constructing calibration curves for each of the target analytes.

2.3. Sample preparation and extraction

2.3.1. Solid oral dosage forms

Twenty five tablets of each solid oral dosage form (Products I–IV) were powdered in a mortar using a pestle. A mass of powder equivalent to one tablet was weighed and dispersed in methanol (25 ml). The mixture was sonicated for 30 min, then manually agitated and the sonication continued for another 30 min prior to centrifugation at 350 × *g* for 15 min. The samples were diluted with appropriate volumes of methanol and water in order to maintain a 50:50 methanol:water solvent ratio and filtered through 0.45 μm PVDF filters before injecting 5 μl.

2.3.2. Hard gelatine capsules

Twenty five capsules (Product V) were emptied, weighed and a mass of powder equivalent to the contents of a single capsule was extracted as described in Section 2.3.1 above.

2.3.3. Soft gelatine capsules

Individual capsules (Product VI) were sliced longitudinally, the contents squeezed out and combined with the soft gel shell containing residual content. Methanol (25 ml) was added and the samples were treated as previously described for the tablets.

2.4. HPLC conditions

HPLC analysis was carried out using a SpectraSYSTEM P2000 pump equipped with an AS 1000 autosampler and UV 1000 variable-wavelength UV detector (all supplied by Thermo Separation Products, Riviera Beach, FL, USA). Separation was achieved isocratically at 45 °C on a 150 mm × 2.0 mm i.d. Luna C_{18} reversed-phase minibore column packed with 5 μm ODS-2. The mobile phase consisted of 0.3% formic acid-acetonitrile (75:25) and was pumped at a flow rate of 0.5 ml/min. The injection volume was 5 μl and the column eluate was monitored on-line with UV detection at 350 nm prior to MS detection.

2.5. MS conditions

Negative ion electrospray ionisation–mass spectrometry (ESI–MS) was performed using a Finnigan MAT LCQ ion trap mass spectrometer (San Jose, CA, USA). The MS parameters for each flavonol were optimized by direct infusion of a mixture of the relevant flavonols at 3 μl/min into the source. Capillary temperature was maintained at 240 °C and the sheath and auxillary gas flow parameters were 80 and 20 arbitrary units, respectively. The spray voltage remained constant for all compounds at 4.5 kV. In addition to detection of the deprotonated molecular ions, collision induced dissociation (CID) was performed in the MS–MS mode and the resulting product ions monitored as fingerprints of each flavonol in the individual commercial products.

2.6. Method validation

2.6.1. Calibration curves

Calibration curves were constructed on three consecutive days by analysis of a mixture containing each of the flavonols at four concentration levels and plotting peak area against the concentration of each reference standard.

2.6.2. Precision and accuracy

The precision and accuracy of the assay were assessed by spiking an amount of powdered material equivalent to the weight of a single tablet of Product I with high, medium and low concentrations of each reference standard. The amount of each analyte was determined in triplicate over 3 days. Since Product I contained a significant amount of rutin (307.3 μg), recovery of rutin at the lowest level was not assessed.

2.6.3. Limits of detection (LOD) and quantification (LOQ)

The limits of detection and quantification were determined by means of serial dilution based on a signal-to-noise (S/N) ratio of 3:1 and 10:1, respectively.

3. Results and discussion

3.1. MS tuning

Full scan negative ion ESI mass spectra were obtained for each of the flavonols by direct infusion of a mixture containing 25 μg/ml of each of rutin, quercitrin, quercetin, kaempferol and isorhamnetin. The deprotonated molecular ions for each of the above mentioned flavonols were observed at *m*/*z* 609, 447, 301, 285 and 315, respectively.

3.2. Collision induced dissociation

In addition to full scan mass spectra, collision induced dissociation was undertaken in the MS–MS mode to yield diagnostic product ion mass spectra, which were characteristic of the structural moieties present in the analytes. While infusing the standard mixture, the collision energy was varied from 10% to 50% and the fragmentation profile investigated for each of the flavonols. The collision energies for rutin, quercitrin, quercetin, kaempferol and isorhamnetin were 32%, 29%, 35%, 47% and 37%, respectively and were chosen so as to obtain product ion mass spectra without total loss of the deprotonated parent molecular ion. Partial integrity of the parent ion aided in the identification of flavonols which differ only in terms of the sugar moeities and therefore have similar fragmentation spectra (see Scheme 1). The fragmentation of rutin gave rise to intense ions at *m*/*z* 301 and *m*/*z* 179 corresponding to the loss of the rutinose unit and subsequent retrocyclization of the C-ring (between bonds 1 and 2) leading to the [1,2] A^- fragment. Quercitrin was observed to lose its terminal rhamnose unit and also produce a product ion at *m*/*z* 301 followed once again by the subsequent retrocyclization pathway to produce the product ion at *m*/*z* 179. Quercetin, in addition to a *m*/*z* 179 fragment, produced product ions resulting from the neutral losses of CO and CO_2 from the C-ring corresponding to the fragments $[M–H–CO]^-$ and $[M–H–CO_2]^-$ at *m*/*z* 273 and *m*/*z* 257, respectively. The ion at *m*/*z* 179 also indicated a retrocyclization fragment, which was consistent with the fragmentation of the flavonols rutin and quercitrin (Scheme 1).

The product ion mass spectrum of kaempferol contained ions at *m*/*z* 257 and *m*/*z* 239 corresponding to the fragments $[M–H–CO]^-$ and $[M–H–CO–H_2O]^-$ respectively. The ejection of CO is notably followed by B-ring rotation and bonding with the A-ring to form the fused ring structure of *m*/*z* 257. The retro-Diels-Alder fragmentation product wherein bonds 1 and 3 undergo scission leading to the formation of the [1,3] A^- ion at *m*/*z* 151 was also evident. The fragmentation pathway of kaempferol, originally proposed by March and Miao [25], is provided by Scheme 2.

Isorhamnetin exhibited fragmentation with the loss of the CH_3 radical from the deprotonated aglycone molecule (*m*/*z* 315), thus producing the ion at *m*/*z* 300 (Scheme 3). These product ion mass spectra were similar to those observed in other mass spectrometry studies of flavonoids [23–25] and thus provided unequivocal identification of the relevant flavonols.

3.3. HPLC–MS–MS

During the chromatographic run, the mass spectrometer was programmed into five segments, which allowed for optimal detection of each flavonol in the MS–MS mode. A typical total ion chromatogram of the five reference standards is shown in Fig. 1 together with their corresponding product ion mass spectra. The elution order of the reference standards were as follows: rutin (1.88 min), quercitrin (2.75 min), quercetin (6.96 min), kaempferol (13.49 min) and isorhamnetin (15.98 min).

3.4. Linearity and range

Calibration data for each compound were obtained using the optimized MS–MS conditions described above. The response profile was determined and observed to be linear for each of the flavonols within the concentration ranges 3–26 μg/ml. The calibration data are presented in Table 1. Each concentration level was analysed in triplicate, once at the start of the day, midway through the analysis and after the sample set was completed. The relative standard deviation (R.S.D.) for all data points was less than 4%.

3.5. Limits of detection and quantification

The limits of detection and quantification were determined by means of serial dilution of the reference standards. The limit of detection was 0.39, 0.40, 1.56, 1.65 and 0.38 μg/ml for rutin, quercitrin, quercetin, kaempferol and isorhamnetin, while their corresponding limits of quantification were 0.78, 0.80, 3.13, 3.30 and 0.76 μg/ml, respectively.

Table 1
Linear ranges and correlation coefficients of calibration curves

Constituents	$y = ax + b$ linear model[a]	Regression (R^2)	Concentrations (x) (μg/ml)			
Rutin	$y = 1E + 07x$	0.9942	3.14	6.28	12.55	25.10
Quercitrin	$y = 2E + 07x$	0.9960	3.21	6.13	12.85	25.70
Quercetin	$y = 6E + 06x$	0.9935	3.13	6.25	12.50	25.00
Kaempferol	$y = 1E + 06x$	0.9968	3.30	6.60	13.20	26.40
Isorhamnetin	$y = 2E + 07x$	0.9957	3.05	6.10	12.20	24.40

[a] y, peak area; x = concentration.

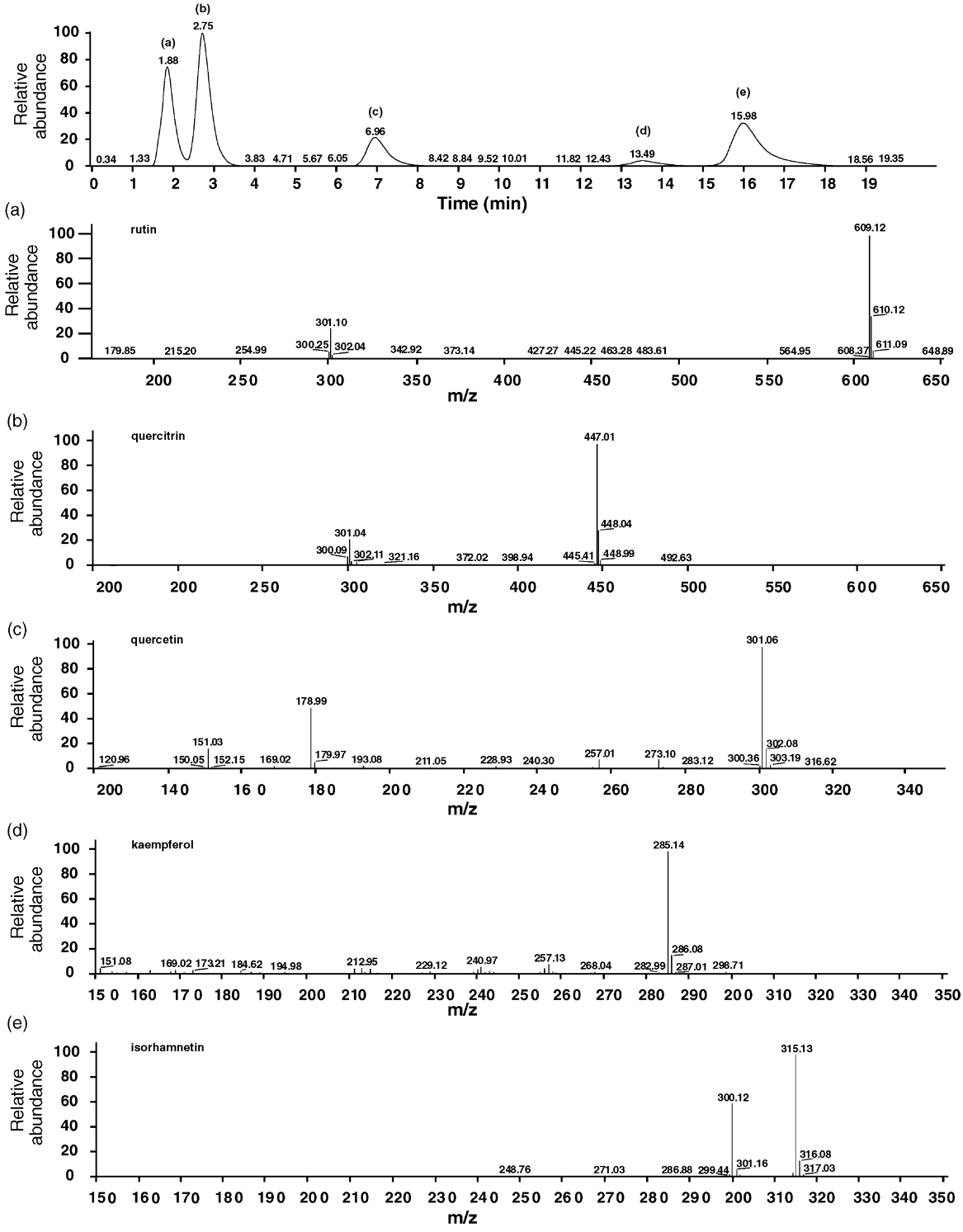


Fig. 1. Elution order of rutin (1.88 min), quercitrin (2.75 min), quercetin (6.96 min), kaempferol (13.49 min) and isorhamnetin (15.98 min) with corresponding fragmentation patterns.

3.6. Precision and accuracy

The reproducibility and accuracy of the method was evaluated by analysing a commercial product (Product I) that was spiked at three concentration levels over two different days. Prior to spiking, the background levels of the flavonols in Product I were determined so as to calculate actual recoveries. Intra-day relative standard deviations were all less than 10% whilst the inter-day R.S.D. ranged between 1 and 16%. Isorhamnetin and quercetin showed the highest R.S.D.;

Table 2
Precision and recoveries of flavonols from Product I

Constituent	Spiking level (μg/500 mg)	Intra-day R.S.D. (%) ($n = 3$)	Inter-day R.S.D. (%) ($n = 6$)	Mean recovery (%) ± S.D. ($n = 6$)
Rutin[a]	–	–	–	–
	312.3	3.0	4.1	94.6 ± 3.9
	450.0	1.2	6.8	90.4 ± 6.2
Quercitrin	131.3	1.5	1.7	93.1 ± 4.9
	287.5	2.8	4.4	93.5 ± 5.7
	425.0	2.4	4.5	90.6 ± 6.5
Quercetin	143.8	4.1	4.4	75.3 ± 1.3
	312.5	1.6	9.5	70.3 ± 7.9
	437.5	3.0	15.9	69.1 ± 10.9
Kaempferol	165.0	6.8	1.3	93.5 ± 8.2
	330.0	3.3	3.7	100.4 ± 3.6
	475.3	2.8	2.2	100.9 ± 2.2
Isorhamnetin	157.3	2.7	15.7	58.8 ± 8.2
	314.5	3.3	11.2	64.1 ± 7.9
	453.0	2.6	5.4	63.7 ± 4.2

[a] Product I contained a quantifiable amount of rutin to fall on the lower linear calibration range. and therefore a low spike was not necessary.

15.61% and 15.32% at the 157 and 437 μg/tablet levels, respectively. Such a high variation can be attributed to solubility problems, which in the case of isorhamnetin has been documented in previous studies [26,27] as a result of its lower polarity. A higher concentration of quercetin stock solution was used and thus the possibility of precipitation of quercetin cannot be discounted and may have contributed to a larger R.S.D. at the highest spiking level. Similarly, whilst recoveries greater than 90% were observed for rutin, quercitrin and kaempferol, lower recoveries were observed for quercetin and isorhamnetin. The results of this experiment are presented in Table 2.

3.7. Analysis of commercial products

The HPLC–MS–MS technique was applied to the analysis of commercially available products of Ginkgo. Fig. 2 illustrates a typical product ion mass chromatogram of a Ginkgo product which can be successfully used as a fingerprint for quality control and hence demonstrates the specificity of this technique in phytochemical analysis.

Table 3 contains the assay results of each of the five flavonols expressed in mg per tablet/capsule. Concerning the aglycones, quantifiable amounts of quercetin, kaempferol and isorhamnetin were found in Product IV whereas Product VI showed quercetin and kaempferol, with a trace of isorhamnetin. None of the other products contained quantifiable amounts of these aglycones. All the products analysed were observed to contain quantifiable amounts of the two flavonol glycosides, rutin and quercitrin. From a product quality aspect, the presence of significant amounts of glycosides are preferable since this indicates minimal degradation during extraction and formulation procedures [18].

During preliminary HPLC–MS analysis of some commercially available Ginkgo products, an unknown compound was found (peak b) to be partially resolved from rutin (Fig. 2). While observing the product ion spectra of rutin and the unknown compound, it was noted that both HPLC peaks yielded deprotonated molecular ions characteristic of rutin (*m*/*z* 609). Investigations through MS–MS revealed that the first peak, eluting at 1.27 min produced a product ion at *m*/*z* 301 resulting from heterolytic cleavage of the rutinoside unit, which also corresponded to the fragmentation profile of the reference standard. The second peak however produced a fragment at *m*/*z* 447 resulting from the loss of glucose $[M–H–Glu]^-$, followed by dehydration $[M–H–Glu–H_2O]^-$ to produce a fragment at *m*/*z* 429. A further product ion observed at *m*/*z* 300 results from homolytic cleavage of the rhamnose unit

Table 3
Flavonol marker content (per product) in six dosage forms purchased at a local pharmacy

Product ($n = 3$)[a]	Rutin[b]	Quercitrin	Quercetin	Kaempferol	Isorhamnetin
I	268 ± 77.3 μg	15.9 ± 0.86 μg	+	–	–
II	1100 ± 9.12 μg	78.6 ± 3.70 μg	+	–	+
III	38.6 ± 0.90 μg	4.55 ± 0.48 μg	+	+	+
IV	6040 ± 109 μg	76.5 ± 1.46 μg	328 ± 6.34 μg	85.0 ± 3.73 μg	42.90 ± 2.52 μg
V	347 ± 15.85 μg	35.5 ± 2.50 μg	–	–	–
VI ($n = 2$)	4750 ± 500 μg	143.7 ± 19.2 μg	224 ± 16.1 μg	113.2 ± 7.17 μg	+

(+) indicates that the marker could be identified but was below the LOQ. (–) indicates that the marker was not detected.
[a] *n*, number of assays conducted per dosage form.
[b] Calculated using selected reaction monitoring (SRM).

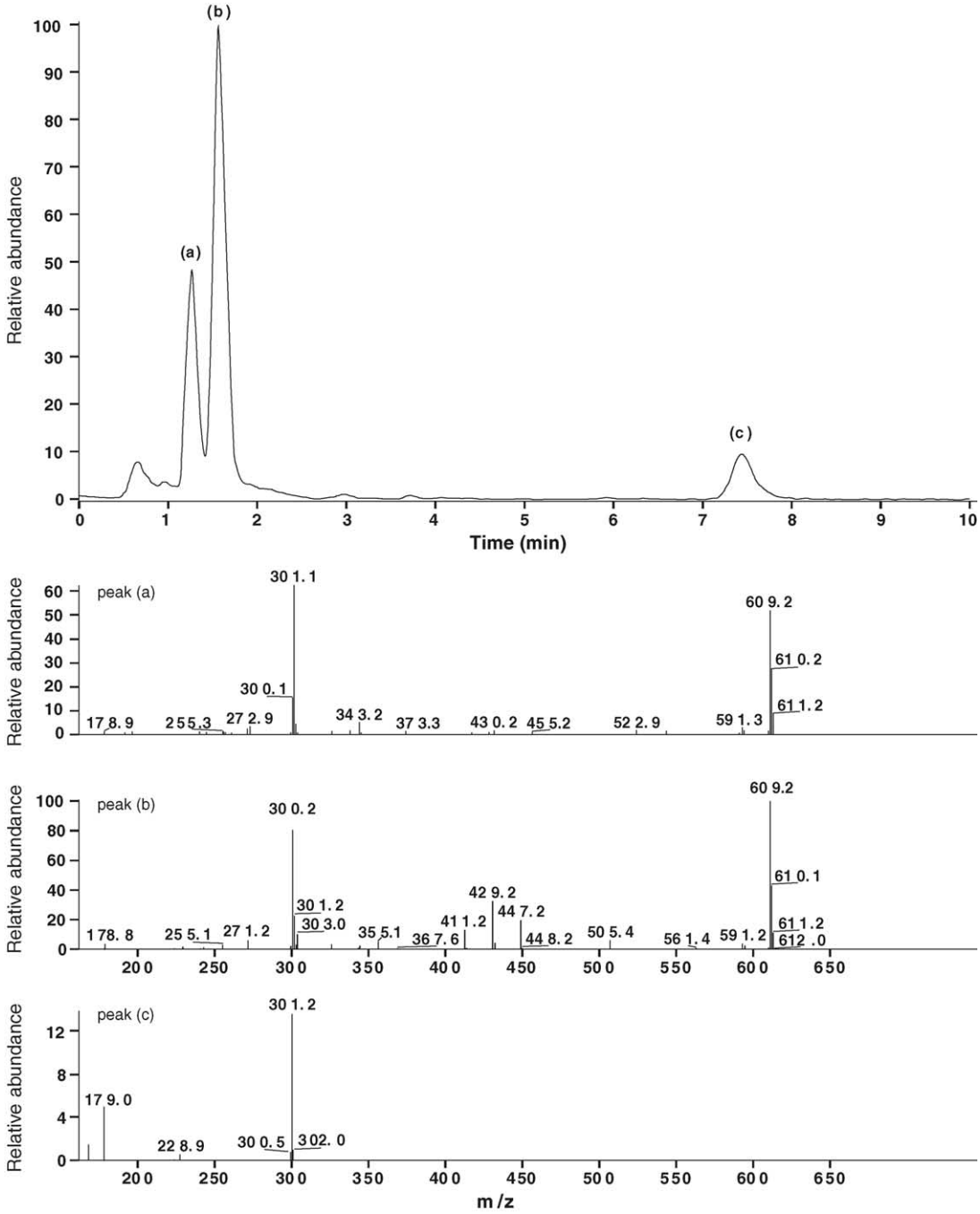


Fig. 2. A typical negative ion TIC of a Ginkgo product with corresponding mass spectra of rutin (a), a quercetin glycoside (b) and quercetin (c).

from the $[M–H–Glu]^-$ fragment. While the first peak provides unequivocal confirmation of rutin, the cleavage pattern of the second peak is suggestive of another quercetin glycoside. In order to improve the resolution between the unresolved peaks, a Phenomenex C_{18} 5 μm (ODS 2) with dimensions 250 mm × 2.00 mm was used. Rutin was then quantified in MS–MS mode with selected reaction monitoring (SRM) of the ions at *m*/*z* 609 and 301. Based on the rutin standard, a separate calibration curve between the range 0.39–25.1 μg/ml was constructed ($R^2 = 0.9964$). In this manner, rutin was selectively and accurately quantified in extracts. The second peak with an $[M - H]^-$ ion at *m*/*z* 609 was also suggestive of a quercetin glycoside. Based on the biochemical pathway of the flavonol glycosides, the most likely positions for glycosylation to take place is at the 3- and 7-hydroxyl group, while the 5-hydroxyl group of quercetin is protected by hydrogen bonding with the adjacent carbonyl group and is therefore never apparently involved in glyco-

sylation [28]. Hence, this would indicate the possibility of one of two quercetin glycoside analogs viz., quercetin 3-*O*-glucoside-7-*O*-rhamnoside or quercetin 3-*O*-rhamnoside-7-*O*-glucoside. The former compound has been found in the leaves of sea buckthorn (*Hippophae rhamnoides* ssp. *Mongolica*) where it was found to be the dominant flavonol in the leaves and accounted for approximately 24% of the flavonoids [29]. The latter compound has been found to be the most abundant glycoside in the leaves of *Ligustrum vulgare* L. (Oleaceae) and the mass spectral pattern matches that observed in the current study [30]. Structural investigations of flavonol glycosides from *H. rhamnoides* recently carried out have demonstrated that the loss of the sugar moiety from C-7 of the aglycone is more favoured than fission of the glycosidic linkage at the C-3 position [31]. Hence, based on literature evidence and the mass spectral pattern, the second peak is suggestive of the 7-*O*-glucoside although further confirmation through NMR spectroscopy needs to be undertaken.

4. Conclusion

A simple, specific, precise, rapid and reproducible LC–MS–MS method has been developed to identify and quantify relevant marker compounds in *Ginkgo biloba* dosage forms. The simultaneous quantitative determination of rutin and quercitrin together with the aglycones, quercetin, kaempferol and isorhamnetin allows the presence of those flavonol glycosides to provide useful stability information which is overrated when samples are hydrolyzed to back calculate flavonol glycoside content. This method also has the advantage that a hydrolysis step, previously used to for the standardization of flavonols in Ginkgo extracts by calculation of the total flavonol glycoside content from the aglycone concentration in extracts, is not required. Tandem mass spectrometry has the additional advantage of ensuring unequivocal identification and quantification of target analytes in a tablet matrix as opposed to UV detection where identification is primarily by retention time of the marker compound. Furthermore, the use of tandem mass spectrometric analysis of Ginkgo extracts revealed, in addition to rutin, the possible presence of other quercetin analogues, quercetin-3-*O*-rhamnoside-7-*O*-glucoside or quercetin-3-*O*-glucoside-7-*O*-rhamnoside. Recent literature findings and the mass fragmentation pattern observed in this study favour the presence of the former analogue although NMR elucidation studies will need to be performed to confirm this speculation. Ginkgo solid oral dosage forms currently available to consumers show major disparities in marker content and product labels contain minimal relevant information. This is indicative that quality control procedures need to be implemented to ensure quality, safety and efficacy of such products.

Acknowledgement

Funding from the South African Medical Research Council is gratefully acknowledged.

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