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Original Article

Analysis of Sterol Glucosides in *Momordica charantia* L. Extracts and Nutraceutical Products

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Abstract

Sterol glucosides were biosynthesised in *Momordica charantia* L. and used as markers for the standardization of *M. charantia* extracts. The sterol glucoside, charantin, used as a marker consisted of equal amounts of two sterol glucosides, namely, 5, 25-stigmastadienol glucoside (**1**) and β-sitosterol glucoside (**2**). Most quantitation methods either mixed up both isomers, namely (**1**) and stigmasterol glucoside (**3**) or both the sterol glucosides (**1**) and (**2**) were not separated in the quantitation methods. The labelling of individual sterol glucosides needs to be clearly stated in nutraceutical products. This study aimed to resolve the separation of the commonly mixed-up sterol glucosides and further validate a high-performance liquid chromatography-photodiode array detector (HPLC-PDA) method for the quantification of individual sterol glucosides in *M. charantia*. The HPLC-PDA instrument was used for method development as it is a universal instrument available in most nutraceutical companies. Sterol glucosides (**1**)-(**3**) were separated with a Zorbax SB-C_{18} column and an isocratic HPLC mobile phase system of 88% methanol in deionized water. The wavelength of the PDA detector was set at 200 to 500 nm with a linearity range of 90 to 300 µg/mL and a good correlation coefficient of $r^2 > 0.99$. The validated method was applied to fresh fruits and nutraceutical products. The sterol glucoside (**1**) is the major constituent in charantin. Hybrid fruits biosynthesised higher content of sterol glucosides compared to non-hybrid fruits. The content of (**1**)-(**3**) in the final nutraceutical products fluctuates and dependent on the source of raw material. Thus, standardization of extracts is essential in nutraceutical production to ensure uniform content of secondary metabolites and reproducible therapeutic effect.

Keywords: Momordica charantia, HPLC, isomer, 5, 25-stigmastadienol glucoside, β-sitosterol glucoside, stigmasterol glucoside

1. Introduction

Momordica charantia L. is a climber plant from the Cucurbitaceae family distributed in the tropical and subtropical regions. The bitter fruits have both the culinary and medicinal uses and are known as bitter gourd [1]. The bitter fresh

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fruits were blended with water and the filtered juice was consumed by diabetic patients [1, 2]. Though the fruit was used traditionally by diabetic patients for glycaemic control, standardisation and the quality control of preparations should be considered before randomised controlled trials to establish the recommended safe and effective dosage for clinical practice [1, 3].

The secondary metabolites biosynthesised in the fruits were affected by the climate changes of different geographical locations, soil conditions, and genetic composition [4, 5, 6]. In addition, the extraction process of the raw material affects the content of secondary metabolites in the extracts [7]. Thus, standardisation of these preparations could ensure a reproducible therapeutic effect. The markers used to standardise the preparations need to be accurately identified and clearly stated on the product label. Mislabelling can lead to disastrous side-effects [8].

The secondary metabolites from this plant consisted of glycosides, saponins, alkaloids, fixed oils, triterpenes, proteins and steroids [9]. Lotlikar and Rao have identified the sterol glucoside, namely charantin, as reducing blood glucose in rabbits [10]. Charantin is comprised of equal amounts of 5, 25-stigmastadienol glucoside (**1**) and β-sitosterol glucoside (**2**) (Figure 1). It has been reported to exhibit both hypoglycaemic [11, 12] and cytotoxic effects [13, 14].

These sterol glucosides were important markers used by various studies on extraction optimisation of *M. charantia* extracts, evaluation of charantin biosynthesis in bitter melon and analysis of charantin content in bitter melon [15, 16, 17].

Various analytical methods were used to assay the content of charantin that is biosynthesised in the fruits. These methods included the high-performance liquid chromatography (HPLC) or high-performance thin-layer chromatography (HPTLC) methods and charantin was identified and reported as a single peak or band without separation although it consisted of both the sterol glucosides (**1**) and (**2**) [15, 16]. In addition, the mixed up between both isomers, namely 5,25-stigmastadienol glucoside (**1**) with stigmasterol glucoside (**3**) caused the inaccurate quantitation of charantin as consisting of both (**3**) and (**2**) [18] instead of (**1**) and (**2**). Thus, this study aimed to develop a method to resolve the separation of (**2**) and the frequently mixed up sterol glucosides, namely (**1**) and (**3**) and further applied the validated method to quantitate these sterol glucosides in *M. charantia* fruits and nutraceutical products. The analytical method could be used to standardize the content of sterol glucosides in *M.charantia* extracts for randomised controlled clinical trials.

2. Materials and Methods

2.1 Sample Preparation

Fresh fruits were collected from UiTM plantation in Puncak Alam campus, Selangor. The age of the hybrid and non-hybrid fruits were 3 weeks old with an average fresh fruit weight of (22.8 ± 4.6) g and (84.3 ± 28.6) g, respectively. Fresh fruits were blended and filtered to produce juice extract. The juice extract was dried using a freeze dryer and weighed. Nutraceutical products in capsule dosage preparations containing *M. charantia* extract were purchased from pharmacy outlets. According to the product label, the recommended dosage for both the nutraceutical products 1 and 2 were two capsules of 500 mg each per day. The content of extracts in nutraceutical products 1 and 2 was 450 mg and 250 mg, respectively. Freeze-dried fruit extract or nutraceutical product samples (100 mg) were extracted in 1mL of mixtures of chloroform and methanol (1:1) solvent, stored in airtight vials and sonicated for 15 minutes. The mixed solution was centrifuged at 3000 rpm for 10 minutes. The extraction procedure was repeated thrice. The combined supernatant was dried under a flow of nitrogen gas. The samples were stored at −20 °C until further analysis. The dried extract was further reconstituted with chloroform and methanol (1:1) v/v to produce a stock solution of 25 mg/mL for sterol glucoside analysis.

2.2. Preparation of Reference Standards

The primary reference standards, namely sterol glucoside (**1**) (93%, IL, USA), sterol glucoside (**2**) (97.8%, Chromadex, USA) and sterol glucoside (**3**) (98%, ChemFaces, China) were dissolved in mixtures of methanol: chloroform (1:1) to prepare a stock solution of 1.5 mg/mL. The sterol glucoside standards identity was reconfirmed with Nuclear Magnetic Resonance (NMR) analysis (Supplementary Data). Mix standard solutions were prepared and diluted to concentrations of 90, 120, 150, 200, 250 and 300 µg/mL.

2.3. HPLC-PDA Method Development

The HPLC (Waters, USA) method with a 717 Plus autoinjector, 600 pump, 2998 photodiode detector monitored from 200−500 nm was developed. The analysis was conducted using a Zorbax SB-C_{18} column (4.6 × 250 mm, 5 µm) (Agilent, US) with an isocratic solvent system consisting of 88% methanol (LiChrosolv® grade, Merck, Germany) and 12% deionized water. The solvent flow rate was 1.2 mL/min and autoinjector volume was set at 10 µL. Column and autosampler temperature were set at 35 °C and 10 °C, respectively. The run time was set for 75 minutes with 10 minutes delay run time to allow other impurities to be eluted out. The peak area of the three standards was integrated at 205 nm.

2.4. Validation of HPLC Analytical Method

Validation of quantitative analysis was carried out with the determination of specificity, linearity and range, accuracy, precision and recovery, the limit of quantitation (LOQ), the limit of detection (LOD) according to the FDA guidance for industry: bioanalytical method validation [19].

The chromatographic specificity was evaluated with the mixed standards (**1**)-(**3**) along

with *M. charantia* extract. The interferences from other impurities were observed from the chromatogram and peak spectra. The linearity curve was established with standard mixtures of (**1**)-(**3**).

The accuracy was calculated by analysing the standard samples against the calibration curves and the measured concentrations were compared with nominal values and expressed as a percentage of nominal values. Six concentration levels of standard samples (90, 120, 150, 200, 250 and 300 µg/mL) were analysed in six replicates for six consecutive days to determine the accuracy and precision. The precision of each sample concentration was expressed as the relative standard deviation (RSD). The precision determined at each concentration level should not exceed 15% RSD except for LOQ not exceeding 20% RSD [19, 20].

The *M. charantia* methanol: chloroform (1:1) extract was spiked with three concentrations of 90, 100 and 110 µg/mL of mixed standards. Recovery was expressed as the amount of (**1**)-(**3**) as a percentage to the theoretical amount present in the extract. The mean value should be within 15% of the nominal value [19]. Quantitation was based on peak area integrated at 205 nm.

3. Results and Discussion

3.1. HPLC-PDA Method Development

Reference standard materials for phytochemical analysis are not easily available. Though some are available commercially, extra precaution is needed to confirm the identity of these reference standards. In the course of this study, we have rejected two charantin reference standards as it didn't match the NMR spectra of triterpenoid glycosides. Thus, we proceeded to purchase individual reference standards and reconfirmed each NMR spectra with published journals.

The separation of the three sterol glucosides, namely, (**1**)-(**3**) was evaluated with a gradient system consisting of methanol and water. Reducing the starting amount of methanol did not separate the impurities in sterol glucoside (**1**) but extended the run time (Figure 2).

Although the starting amount of methanol was reduced to 87% (Figure 2), a shoulder peak was still observed at the peak (**1**). The second and third standard peaks, namely, (**2**) and (**3**) eluted at a longer retention time. Thus, an isocratic mobile phase system consisting of 88 to 95% methanol in deionised water was evaluated. When the amount of methanol was reduced from 95% to 88 % of methanol in water, the co-eluting peak in (**1**) was resolved. The isocratic system of 88% methanol in deionised water provided a baseline separation of sterol glucoside (**1**) from its impurities (Figure 3).

The mobile phase flow rate was increased to 1.2 mL/min to reduce the elution time without exceeding the high-pressure limit of the column. Replacing the Zorbax SB-C_{18} column to a core-shell column created very high backpressure. Therefore, Zorbax SB-C_{18} column was maintained as the column used for the separation. A shorter 15 cm column was not able to resolve the separation of isomers, namely (**1**) and (**3**).

The elution sequence of mixed standards was monitored at 205 nm and the sterol glucoside, (**1**), (**3**) and (**2**) were eluted at the retention time

(Rt) of 45.8 min, 52.8 min and 63.8 min, respectively (Figure 4).

In addition to retention time, the spectra of peaks provided additional information for the peak confirmation (Figure 4). Due to the complexity of plant extract matrix, most natural product chromatographic profile requires longer run time [20, 21]. Though the runtime was longer, the assay method can easily be reproduced as most labs have an HPLC with a diode array or ultraviolet detector without investing in new instruments e.g. UHPLC or LCMS. The two sterol glucosides in charantin, namely (**1**) and (**2**) were well separated at a longer run time as compared to a single peak as reported by Pitipanapong et al. [15]. The charantin single peak was eluted at Rt of approximately 13 min with an isocratic mobile phase system of methanol in water (100:2) at 1 mL/min separated with an ODS-3 column (5 µm, 4.6 x 250 mm ID, Inertsil, Japan) [15]. Thus, the present developed method consisting of an isocratic mobile phase system of 88% methanol in water was able to resolve the separation of both the sterol glucosides (**1**) and (**2**) in charantin.

3.2. Method Validation

The calibration curve of peak area absorbance versus concentration for sterol glucosides (**1**)-(**3**) was linear over the concentration range between 90−300 µg/mL. The linear regression equation of sterol glucoside (**1**) was y=1175x + 14745 with a correlation coefficient, r^2 of 0.999. While the linear concentration range for **(2)** and (**3**) was fitted over linear regression equations of y=2268x − 593.4 and y=2196x − 5278, respectively, and both have a correlation coefficient of $r^2 = 0.998$.

The lower limit of quantitation, LLOQ was 90 µg/mL with a signal to noise ratio of ten established for sterol glucosides (**1**)-(**3**). Limit of detection (LOD) for (**1**)-(**3**) was 80 µg/mL with a signal to noise ratio of less than three. The three reference standards have similar parent structure with a simple alkene chromophore at C-5 and C-6. The electronic transition $\pi \rightarrow \pi^*$ of an alkene chromophore caused the absorption to occur in the far ultraviolet of 204 nm. Without conjugation of the alkene chromophore, the absorption of a simple alkene has low sensitivity towards ultraviolet absorption [22].

The measurement of within-day and between-day precision were utilized to assess the repeatability and reproducibility of the developed method. The within-day precision (*n*=6) for sterol glucosides (**1**)-(**3**) for concentrations of 90, 120, 150, 200, 250 and 300 µg/mL were between 1.2 to 2.9%, 1.0 to 2.1% and 0.9 to 2.1%, respectively (Table 1).

The between-day precision for concentration of sterol glucosides (**1**)-(**3**) were between 0.4 to 2.0%, 0.9 to 1.6% and 1.1 to 2.1%, respectively (Table 1). In addition, the within-day retention time precision of sterol glucosides (**1**)-(**3**) were 0.3% to 0.7%, 0.2% to 0.4% and 0.3 to 0.5%, respectively (Table 2).

The between-day precision for retention time of sterol glucosides (**1**)-(**3**) were between 0.3% to 0.8%, 0.1% to 0.5% and 0.2% to 0.5%, respectively. Accuracy and precision values obtained were within the recommended value of ±15%. Retention time is an important parameter

to confirm the identity of the peaks in the chromatogram.

The accuracy of sterol glucosides (**1**)-(**3**) were 87.0–96.7%, 104.0–113.1% and 86.0–89.5%, respectively measured at concentrations of 90, 100 and 110 µg/mL (Table 3).

The accuracy of sterol glucoside (**1**) for the concentrations of 90, 100 and 110 µg/mL were 87.0% with 2.0% RSD, 94.9% with 4.1% RSD and 96.7% with 3.1% RSD, respectively. The accuracy of sterol glucoside (**2**) were 104.0% with 3.0% RSD, 108.1% with 2.6% RSD and 113.1% with 3.1% RSD, respectively. Meanwhile, the accuracy of sterol glucoside (**3**) for concentrations of 90, 100 and 110 µg/mL were 88.6% with 2.1% RSD, 89.5% with 3.3% RSD and 86.0% with 2.3% RSD, respectively.

3.3. Content of Sterol Glucosides in Samples

The hybrid fruits yielded a higher amount of both the sterol glucosides (**1**) and (**3**) (Table 4). The content of both sterol glucosides (**1**) and (**2**) which is represented as charantin was between 0.020-0.021 % and 0.067-0.072 % in non-hybrid and hybrid fruits, respectively (Table 4). These amounts have a similar range as the Japanese and Philippines cultivars with 0.0087-0.0711% and 0.0029-0.0235%, respectively [17]. The *M.charantia* fruit from Thailand used to study the pressurized liquid extraction of charantin was reported to contain 0.0126 % [15]. Thus, the fluctuations of sterol glucosides content in the raw material will affect the content in the final nutraceutical products. Standardisation of these sterol glucosides in the final nutraceutical preparations is essential to have a reproducible therapeutic effect.

The content of sterol glucoside (**1**) and (**3**) in hybrid fruits were three- and four-fold more than non-hybrid fruits, respectively. The lowest content was sterol glucoside (**2**) (Table 4). Although charantin consisted of both the (**1**) and (**2**), the higher amount was contributed from (**1**).

Both the nutraceutical products 1 and 2 have almost similar content of sterol glucosides (**2**) and (**3**). The content of (**1**) in nutraceutical product 1 is six-fold more than the content in nutraceutical product 2. All the samples evaluated contained a higher content of (**1**) compared to (**3**), except nutraceutical product 2 having a higher content of (**3**). The content of secondary metabolites (**1**)-(**3**) in the extracts was affected by the climate changes of different geographical locations, soil conditions and genetic composition and extraction process [4, 5, 6, 7]. Thus, the total content of sterol glucosides in nutraceutical product 1 is almost twice the amount compared to nutraceutical product 2 (Table 4). The differences in its content may affect the therapeutic effect of these nutraceutical products. Thus, standardization of extracts used for nutraceutical manufacturing is essential to ensure the therapeutic effect is reproducible.

The chromatogram of the nutraceutical product 2 samples showed an unknown peak (RT=61 min) closed to peak (**2**) (Figure 5).

For further confirmation of the unknown peak, the sample was spiked with mixed standards (Figure 5) and two peaks were observed (Rt=61 and 64 min). This confirmed the unknown peak was different from sterol glucoside (**2**). Thus, it is essential to fully resolve the separations of all peaks around the

region of peaks (**1**)-(**3**) to avoid overlapping peaks. In medicinal herbs and food plants, steroidal saponins are of interest because of their potential pharmacological activity and/or toxicity in animals. The correct identification of these steroidal saponins is essential as it can cause liver injury [23].

4. Conclusion

A new isocratic HPLC method is developed and validated to quantify individual sterol glucosides (**1**)-(**3**). The sterol glucoside (**1**) is the major constituent in charantin. Hybrid fruits produced higher content of sterol glucosides compared to non-hybrid fruits. The content of (**1**)-(**3**) in the final nutraceutical products fluctuates and dependent on the source of raw material. Thus, standardization of extracts is essential in nutraceutical production to ensure uniform content of secondary metabolites and reproducible therapeutic effect.

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References

[1] Saeed F, Afzaal M, Niaz B, Arshad, MA, Tufail T, Hussain MB and Javed A. Bitter melon (*Momordica charantia*): a natural healthy vegetable. *Int. J. Food Prop.* (2018) 21: 1270-90.

[2] Jiang S, Xu L, Xu Y, Guo Y, Wei L, Li X and Song W. Antidiabetic effect of *Momordica charantia* saponins in rats induced by high-fat diet combined with STZ. *Electron J. Biotechn.* (2020) 43: 41-7.

[3] Ooi CP, Yassin Z and Hamid TA. *Momordica charantia* for type 2 diabetes mellitus. *Cochrane Database Syst. Rev.* (2012) 15: CD007845.

[4] Sharma S, Walia S, Rathore S, Kumar P and Kumar R. Combined effect of elevated CO_2 and temperature on growth, biomass and secondary metabolite of *Hypericum perforatum* L. in a western Himalayan region. *J. Appl. Res. Med. Aromat.* (2020) 16: 100239

[5] Alinejad S, Sarabi V, Bakhtvari ARS and Hashempour H. Variation in physiological traits, yield and secondary metabolites of jimsonweed (*Datura stramonium* L.) under different irrigation regimes and nutrition systems. *Ind. Crops Prods.* (2020) 143: 111916.

[6] Zhang Y, Wang L, Wei K, Ruan L, Wu L, He M, Tong H and Cheng H. Differential regulatory mechanisms of secondary metabolites revealed at different leaf positions in two related tea cultivars. *Sci. Hortic.* (2020) 272: 109579.

[7] Li W, Zhao F, Pan J and Qu H. Influence of ethanol concentration of extraction solvent on metabolite profiling for *Salviae Miltiorrhizae* Radix et Rhizoma extract by ^{1}H NMR spectroscopy and multivariate data analysis. *Process Biochem.* (2020) 97: 158-67.

[8] Navarro V, Avula B, Khan I, Verma M, Seeff L, Serrano J, Stolz A, Fontana R and Ahmad J. The contents of herbal and dietary supplements implicated in liver injury in the United States are frequently mislabeled. *Hepatol. Commun.* (2019) 3: 792-794.

[9] Grover JK and Yadav SP. Pharmacological actions and potential uses of *Momordica charantia*: a review. *J. Ethnopharmacol.* (2004) 93: 123–32.

[10] Lotlikar MM and Rao MR. Pharmacology of a hypoglycaemic principle isolated from the fruits of *Momordica charantia* Linn *Indian J. Pharm.* (1966) 28: 129−33.

[11] Kumar R, Balaji S, Uma TS and Sehgal PK. Fruit extracts of *Momordica charantia* potentiate glucose uptake and up-regulate Glut-4, PPAR and PI3K. *J. Ethnopharmacol.* (2009) 126: 533−7.

[12] Wang HY, Kan WC, Cheng TJ, Yu SH, Chang LH and Chu JJ. Differential anti-diabetic effects and mechanism of action of charantin-rich extract of Taiwanese *Momordica charantia* between type 1 and type 2 diabetic mice. *Food Chem. Toxicol*. (2014) 69: 347−56.

[13] Manoharan G, Cummings E and Singh J. Effects of crude water-soluble extract of *Momordica charantia* on viability, caspase activity, cytochrome-c release and on cytosolic calcium levels in different cancer cell lines. *Cancer Cell & Microenvironment* (2014) 1: 1−10.

[14] Yung MMH, Ross FA, Hardie DG, Leung THY, Zhan J, Ngan HYS and Chan DW. Bitter melon (*Momordica charantia*) extract inhibits tumorigenicity and overcomes cisplatin-resistance in ovarian cancer cells through targeting AMPK signalling cascade. *Integr. Cancer Therap.* (2016) 15: 376−89.

[15] Pitipanapong J, Chitprasert S, Goto M, Jiratchariyakul W, Sasaki M and Shotipruk A. New approach for extraction of charantin from *Momordica charantia* with pressurized liquid extraction. *Sep. Purif. Technol.* (2007) 52: 416−22.

[16] Cuong DM, Jeon J, Morgan AMA, Kim C, Kim JK, Lee SY and Park SU. Accumulation of charantin and expression of triterpenoid biosynthesis genes in bitter melon (*Momordica charantia*). *J. Agricul. Food Chem.* (2017) 65: 7240−9.

[17] Kim YK, Park WT, Uddin MR, Kim YB, Bae H, Kim HH, Park KW and Park SU. Variation of charantin content in different bitter melon cultivars. *Asian J. Chem.* (2014) 26: 309−10.

[18] Desai S and Tatke P. Charantin: An important lead sterol glucoside from *Momordica charantia* for the treatment of diabetes. *J. Pharmacog. Phytochem.* (2015) 3: 163−6.

[19] FDA, Guidance for Industry: Bioanalytical Method Validation. 2018. US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CMV). Available from: URL: https://www.fda.gov/downloads/drugs/guidances/ucm070107.pdf

[20] Man F and Choo CY. HPLC-MS/MS method for bioavailability study of bruceines D & E in rat plasma. *J. Chromatogr. B* (2017) 1063: 183-8.

[21] Choo CY, Mohd Nor NA, Sahidan NS, Latiff A and Hitotsuyanagi Y. Biomarkers identification of Lycopodiaceae and Huperziaceae species from peninsular Malaysia with HPLC chromatographic profiling and partial least square analysis. *Sep. Sci. Plus* (2018) 1:660–8.

[22] Morrison RT and Boyd RN. *Organic Chemistry*, Sixth Edition. Prentice Hall Publisher, NY, USA. (1992) 599-600.

[23] Su D, Liao Z, Feng B, Wang T, Shan B, Zeng Q, Song J and Song Y. *Pulsatilla chinensis* saponins cause liver injury through interfering ceramide/sphingomyelin balance that promotes lipid metabolism dysregulation and apoptosis. *Phytomed.* (2020) 76: 153265.

Tables:

Table 1. Within-day and between-day precisions on concentration of sterol glucosides (**1**)–(**3**).

		Within-day		Between-day	
Compounds	**Concentration (μg/mL)**	**Mean measured concentration (μg/mL)**	**RSD (%)**	**Mean measured concentration (μg/mL)**	**RSD (%)**
(1)	300.0	281.6	1.6	282.1	0.9
	250.0	235.8	1.2	235.9	0.4
	200.0	194.9	1.7	194.4	1.8
	150.0	150.0	2.9	150.0	1.3
	120.0	120.5	1.9	118.8	1.6
	90.0	91.6	2.1	90.5	2.0
(2)	300.0	288.3	0.9	288.8	0.9
	250.0	240.2	1.7	241.6	1.1
	200.0	196.6	1.0	196.2	1.4
	150.0	150.0	1.4	150.0	1.0
	120.0	115.4	2.1	114.8	1.6
	90.0	83.2	2.0	82.7	1.3
(3)	300.0	293.3	1.1	289.4	1.1
	250.0	242.5	1.2	239.9	1.5
	200.0	196.6	2.1	195.0	2.1
	150.0	150.0	1.8	150.0	1.4
	120.0	116.4	1.0	116.0	1.4
	90.0	83.2	2.0	81.6	2.0

Table 2. Within-day and between-day precisions on retention time of sterol glucosides (**1**)–(**3**).

		Within-day		Between-day	
Compounds	**Concentration (μg/mL)**	**Mean retention time (min)**	**RSD (%)**	**Mean retention time (min)**	**RSD (%)**
(1)	300.0	45.3	0.4	45.5	0.3
	250.0	45.5	0.4	45.3	0.8
	200.0	45.6	0.7	45.7	0.4
	150.0	45.4	0.5	45.5	0.3
	120.0	45.3	0.7	45.5	0.3
	90.0	45.2	0.3	45.4	0.4
(2)	300.0	64.0	0.2	64.0	0.1

	250.0	63.9	0.3	64.0	0.3
	200.0	64.4	0.4	64.5	0.1
	150.0	64.0	0.3	64.2	0.2
	120.0	63.8	0.4	64.3	0.5
	90.0	63.6	0.3	64.2	0.4
(3)	300.0	52.6	0.4	52.7	0.3
	250.0	52.4	0.3	52.5	0.5
	200.0	52.8	0.5	52.8	0.2
	150.0	52.5	0.3	52.6	0.2
	120.0	52.6	0.4	52.8	0.5
	90.0	52.3	0.5	52.6	0.3

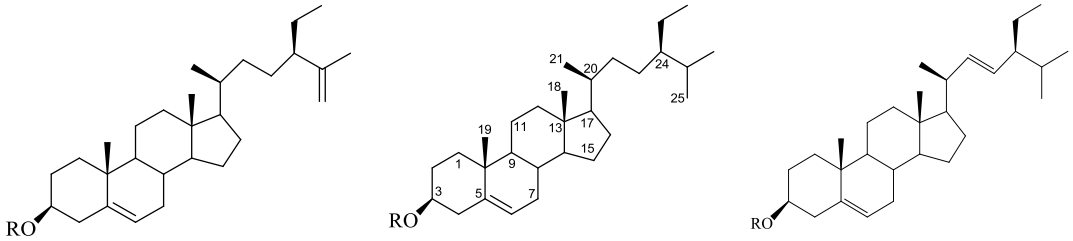
Table 3. Accuracy of sterol glucosides (**1**)–(**3**).

Compounds	**Concentrations (µg/mL)**	**Accuracy (mean±SD, %)**	**RSD (%)**
(1)	90.0	87.0±1.8	2.0
	100.0	94.9±3.8	4.1
	110.0	96.7±3.0	3.1
(2)	90.0	104.0±3.1	3.0
	100.0	108.1±2.8	2.6
	110.0	113.1±3.5	3.1
(3)	90.0	88.6±1.9	2.1
	100.0	89.5±2.9	3.3
	110.0	86.0±2.0	2.3

Table 4. Contents of sterol glucosides (**1**)-(**3**) in *M. charantia* samples.

Samples	**(1)**	**(2)**	**(3)**
Non-hybrid fruit 1(%)	0.017±0.009	<0.003	0.005±0.001
Non-hybrid fruit 2(%)	0.018±0.007	<0.003	0.006±0.002
Hybrid fruit 1(%)	0.064±0.008	<0.003	0.023±0.007
Hybrid fruit 2(%)	0.069±0.025	<0.003	0.018±0.028
Nutraceutical product 1 (ppm)	3143.1±26.2	49.2±1.0	600.5±4.0
Nutraceutical product 2 (ppm)	515.9±6.2	46.7±0.6	729.8±0.2

Figures:



R=glucose

5,25-Stigmastadienol glucoside (**1**) β-Sitosterol glucoside (**2**) Stigmasterol glucoside (**3**)

Figure 1. Chemical structures of sterol glucosides.

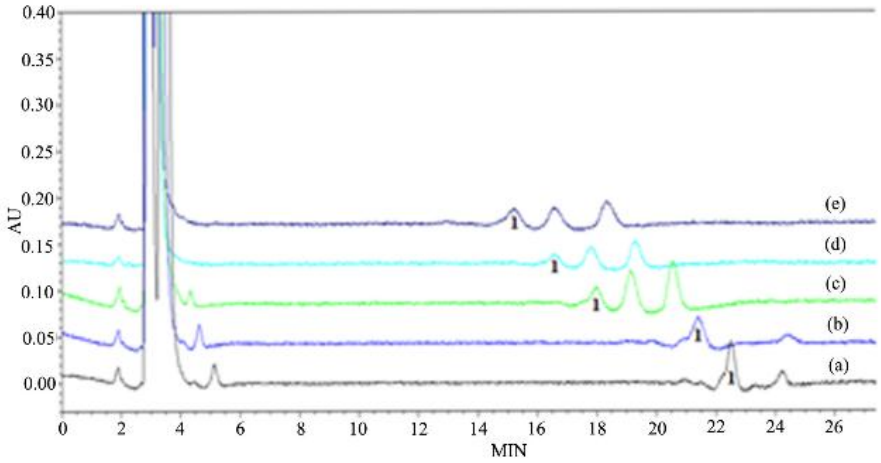


Figure 2. Chromatogram of (**1**) eluted with a gradient mobile phase consisted of a) 87−100%; b) 90−100%; c) 92−100%; d) 93−100%; e) 94−100% methanol and water.

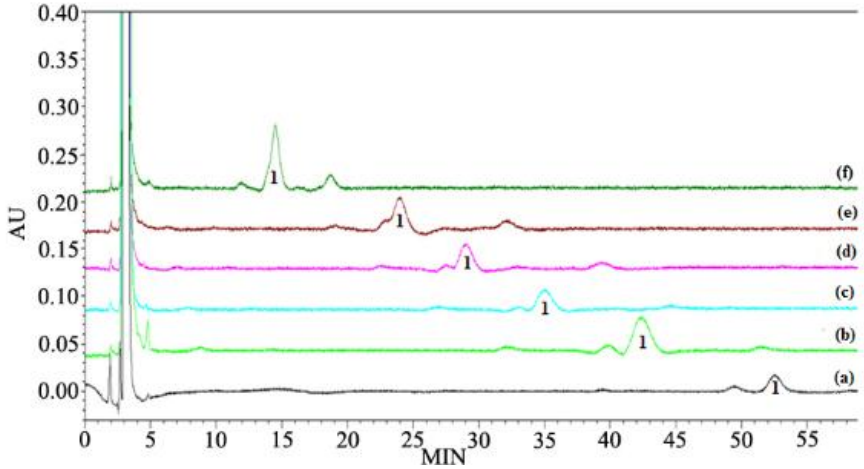


Figure 3. Chromatogram of (**1**) eluted with isocratic mobile phase of a) 88% b) 89%; c) 90%; d) 91%; e) 92%; f) 95% of methanol in water.

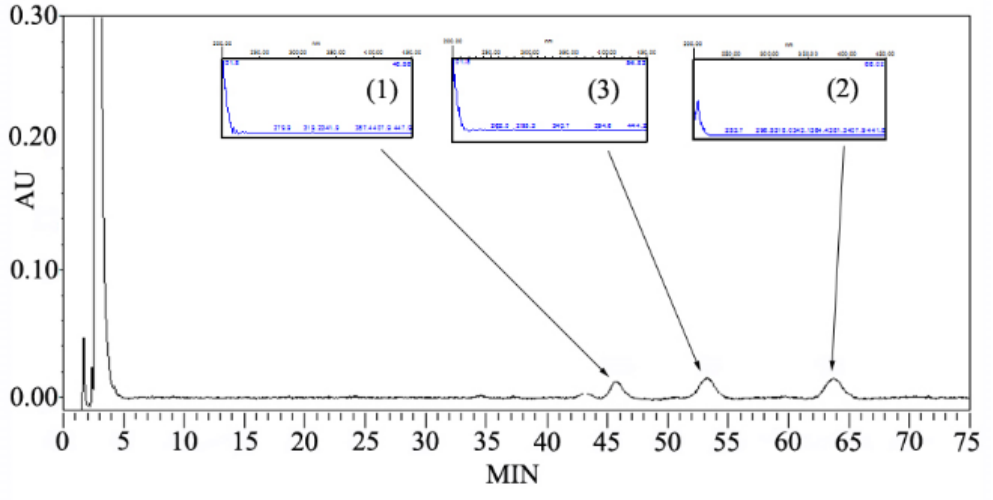


Figure 4. Chromatogram and spectra of mixed standards (**1**)-(**3**).

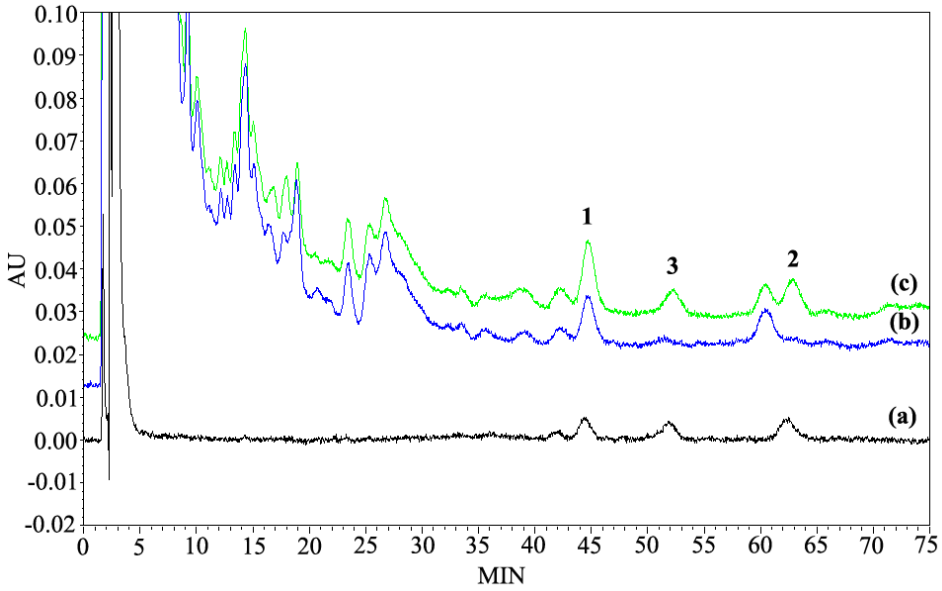


Figure 5. Chromatogram of mixed standards (a), nutraceutical product (b) and nutraceutical product spiked with mixed standards (c).