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Determination of β-Sitosterol in *Alchemilla caucasica* Buser by GC-MS Method

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Abctract
In this study, it was aimed to develop a method for the determination of β-sitosterol by gas chromatography-mass spectrometry method in *Alchemilla caucasica* Buser. For β-sitosterol, selected ion monitoring and electron ionization modes were used. Calibration linearity of the devoloped method was obtained at 1-100 µg/mL concentration for β-sitosterol. The intra-day and inter-day precision value for β-sitosterol was less than 4.99%. The accuracy of the method was observed to be better than 4.60%. The detection and quantification limits for β-sitosterol were found 0.05 and 0.15 µg/mL, respectively. The amount of β-sitosterol in *A. caucasica* plant was found to be 22.6 µg/1.0 mg with percent recovery 2.26% for β-sitosterol. Also, this method can be used for the determination of β-sitosterol in *A. caucasica* plant.

Keywords: *Alchemilla caucasica*, β-sitosterol, GC-MS, Validation

Alchemilla caucasica'da β-Sitosterol'ün GC-MS Yöntemi ile Belirlenmesi

Öz
Bu çalışmada *Alchemilla caucasica* bitkisinde β-sitosterol'ün gaz kromatografisi-kütle spektrometri yöntemiyle belirlenmesi için bir yöntem geliştirmesi amaçlandı. β-sitosterol için, seçilmiş iyon görüntüleme ve elektron iyonizasyon modları kullanıldı. Geliştirilen yöntemin doğrusal aralığı β-sitosterol için 1-100 µg/mL olarak elde edildi. β-sitosterol için gün-içi ve günler-arası kesinlik değerinin %4.99'dan daha düşüktü. Yöntemin doğruluğunun %4.60'dan daha iyi olduğu görüldü. Gözlenebilme ve miktar tayin sınırları β-sitosterol için sırasıyla 0.05 ve 0.15 µg/mL olarak bulundu. *A. caucasica* bitkisinde β-sitosterol miktarı 2.26%'lık geri kazanımıyla 22.6 µg/1.0 mg olarak bulundu. Öyle ki bu yöntem, *A. caucasica* bitkisinde β-sitosterol'ün tayini için de kullanılabilir.

Anahtar Kelimeler: *Alchemilla caucasica*, β-sitosterol, GC-MS, GC-MS, Validasyon

1. Introduction

Herbal treatments play an important role in primary health care as therapeutic solutions in developing countries (Jonathan et al, 2007). They serve as source of medicine and an important component in health care system (Kathirvel and Sujatha, 2016). This stems from the fact that aside from providing vitamins and minerals, plants contain phytochemicals which are secondary metabolites. These phytochemicals have various bioactivities such as being antioxidants, antinutritional or cytotoxic. Free radicals are compounds that cause diseases such as heart disease, liver disease, cancer and damage the body tissues. Antioxidants are substances that reduce or eliminate the damage of free radicals (Awiram, 2000; Owen et al., 2000). The antinutritional activity of a bioactive compound is based on its ability to inhibit the absorption and utilization of nutrients. In recent years there has been increased interest in studies on bioactive components found in plants and their beneficial effects.

Since ancient times, medical plants have traditionally been used. According to the World Health Organization, 74 % of the

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herbal medicines are used in modern medicine and also traditional treatment and modern medical practices are related to each other (Mukherjee, 2002; Kumar and Parmar, 2003).

Alchemilla species belonging to Rosaceae family are locally known as "aslanpençesi" in Turkey. This species include fatty acids, esters, aldehydes, terpenes, hydrocarbons, mainly phenolic compounds, including flavonoids and tannins (Ho et al., 1995; Zuo et al, 2002; Falchero et al, 2009; Kaya et al, 2012). *Alchemilla* species are traditionally used in the treatment of sore throat, wound, bleeding, gynecological diseases, nausea and vomiting (Makau et al, 2013). It is believed that these activities may be due to the phenolic compounds (tannins, flavonoids, etc.) found in the *Alchemilla* plants (Jonadet et al., 1986; Filipek, 1992, Schimmer and Lindenbaum, 1995). *Alchemilla caucasica* is a member of this genus. This plant has short, erect flowering stems, wide and reniform leaves (Davis, 1972).

Sterols are important components for eukaryotes and play a role in plant cell membranes. Plant sterols have many physiological activities (Matthaus and Ozcan, 2014). Among all phytochemicals, β-sitosterol is a main phytosterol found in many plants. It has been reported to show anti-inflammatory, antineoplastic, antipyretic, and immunomodulating activity (Fraile et al., 2012; Awad et al, 2000).

There is no literature, which was performed the determination of β-sitosterol in *A. caucasica* by GC-MS. Therefore, a new and simple GC-MS method has been validated using ICH guidelines (Guidance for Industry: Bioanalytical Method Validation) for the determination and quantification of β-sitosterol form dried whole plant powder of *A. caucasica.*

The HPLC and GC-MS methods have an important place. Therefore, these analytical techniques are widely used. The high-resolution capillary GC-MS method has excellent precision and accuracy compared to HPLC (Yılmaz et al, 2009).

Literature survey revealed that some of the related methods were reviewed. In a reported method (Martelanc et al, 2009) chromatographic separation of twelve compounds including α-amyrin, lupeol, and β-sitosterol from *Brassica oleracea* L., *Solanum lycopersicum* L., *Rosmarinus officinalis* L., *Salvia officinalis* L., and *Quercus robur* L. was studied. The study described a combination of two RP-HPTLC methods for a qualitative determination of twelve phytochemicals and evaluation of their presence in different plant extracts. In the study, RP-HPTLC was used to analyse the phytochemicals. Experiment was performed on RP-HPTLC plates, using the combination of two mobile phases to isolate compounds; these were further identified using RP-HPLC method. The reported methods were only used for qualitative screening and identification of these compounds. Also, capillary GC was used for quantitation of α-amyrin, β-sitosterol, and lupeol from aerial part of *Justicia anselliana* (Kpoviéssi et al., 2008).

A GC-MS method was reported for analysing compounds in *Salvia bicolor* Desf. extract. β-sitosterol and lupeol were detected. The retention time observed was 41.04 min for β-sitosterol and 41.5 min for lupeol (Ibrahim, 2012). Both methods were time consuming. Therefore, in the present research work, in order to standardize the plants with these markers, a new, simple, accurate and precise GC-MS method for β-sitosterol from the extract of whole plant powder of *A. caucasica* plant was developed. Also, β-sitosterol has also been quantified.

2. Material and Method

2.1. Chemical and reagents

β-sitosterol (95.0% purity) and methanol (99.8% purity) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. GC-MS system

The analysis were carried out on Agilent 7820A gas chromatography system. In this study, HP-5 MS column (0.25 μm, 30 m × 0.25 mm I.D.) was used. The temperatures of the detector, inlet and transfer line were 300, 250 and 250 ^{o}C, respectively.

2.3. GC-MS conditions

In this study, different temperature programs were used for the method. The end of these programs, initial temperature was 150 ^{o}C, held for 2 min, increased to 220 ^{o}C at a rate of 30 ^{o}C /min, held for 2 min, and finally to 300 ^{o}C at a rate of 20 ^{o}C /min and held for 9.7 min. The injector volume was 1 μl in splitless mod. GC-MS carrier helium gas was at 1.5 mL/min flow rate.

2.4. Plant materials

A. caucasica was collected from Konaklı Mountain (Erzurum province, Turkey) in June, 2016. The drying process of the plants and all the remaining studies were carried out in Ataturk University, Faculty of Pharmacy Laboratory.

2.5. Extraction procedure

Methanol extracts were prepared from the dried whole plant of *A. caucasica*. In this method, methanol is added in a volume of about 3 times that of the plants on 100 g of powder-dried plants and allowed to shake on a shaker for three days at room temperature. At the end of three days, the liquid layer is filtered. The obtained filtrate is put into a rotary evaporator and the methanol is removed. After all of the methanol is removed, the whole dry extracts are scraped with a spatula and weighed. These are stored at + 4 ^{o}C until use in study. The dry extracts were then serially diluted with methanol then 1 μl solution was employed to GC-MS system.

2.6. Standards and quality control samples

Reference standard stock solution of β-sitosterol were prepared by dissolving the accurately weighed reference compounds in methanol to give a final concentration of 1000 μg/mL. The solution was then serially diluted with methanol to achieve standard working solutions at concentrations of 1, 2.5, 5, 10, 25, 50 and 100 μg/mL for β-sitosterol. Structural formula of β-sitosterol is shown in Figure 1.

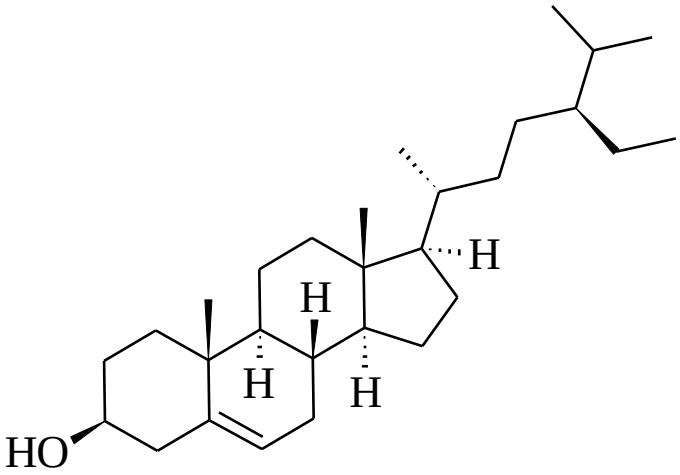

Figure 1. Chemical formula of β-sitosterol

All solutions were stored at 4 ^{o}C and were brought to room temperature before use. The quality control (QC) solutions were prepared by adding aliquots of standard working solution of final concentrations of 15, 50 and 75 μg/mL for β-sitosterol.

2.7. Identification of components

Identification was based on the molecular structure, molecular mass and calculated fragments. Interpretation on mass spectrum GC-MS was conducted using the database on National Institute Standard and Technology having more than 62,000 patterns. The name, molecular weight and structure of the components of the test materials were ascertained. The relative percentage amount of each component was calculated by comparing its average peak area to the total areas. The spectrum of the unknown component was compared with the spectrum of the component stored in the National Institute of Standards and Technology

Library Version (2005), Software, Turbomass 5.2.

3. Results and Discussion

3.1. The method validation

Parameters such as specificity, linearity, precision, accuracy, limit of detection, limit of quantification and stability parameters were investigated according to the ICH (Guidance for Industry: Bioanalytical Method Validation).

3.1.1. Specificity

The specificity of the method was investigated for the β-sitosterol and excipients. No derivatization was performed prior to GC-MS analysis. In this study, selected ion monitoring was used for β-sitosterol. The scan mass spectrum is shown in Figure 2. The molecular weight of β-sitosterol is 414. It was identified as β-sitosterol with molecular formula of $C_{29}H_{50}O$ and base peak at m/z 145. (Igwe et al., 2013). To increase the sensitivity and performance of the gas chromatographic separation, the fragment ion base peak was selected as base peak (m/z 145). β-sitosterol retention time for the method was 14.9 min (Figure 3).

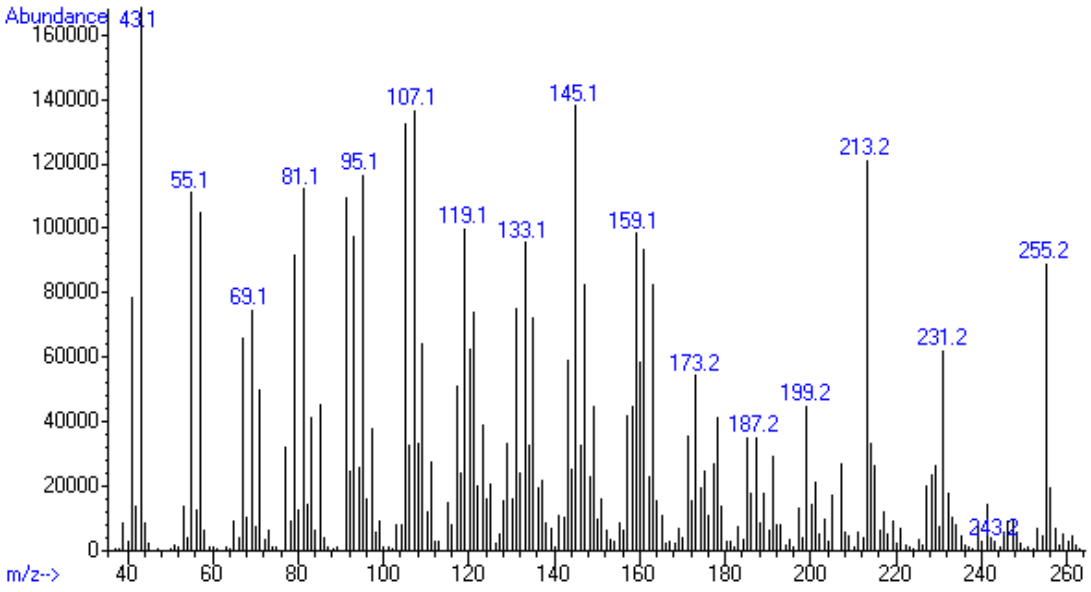

Figure 2. β-sitosterol mass spectrum, (The temperatures of the detector, inlet and transfer line were 300, 250 and 250 °C, respectively).

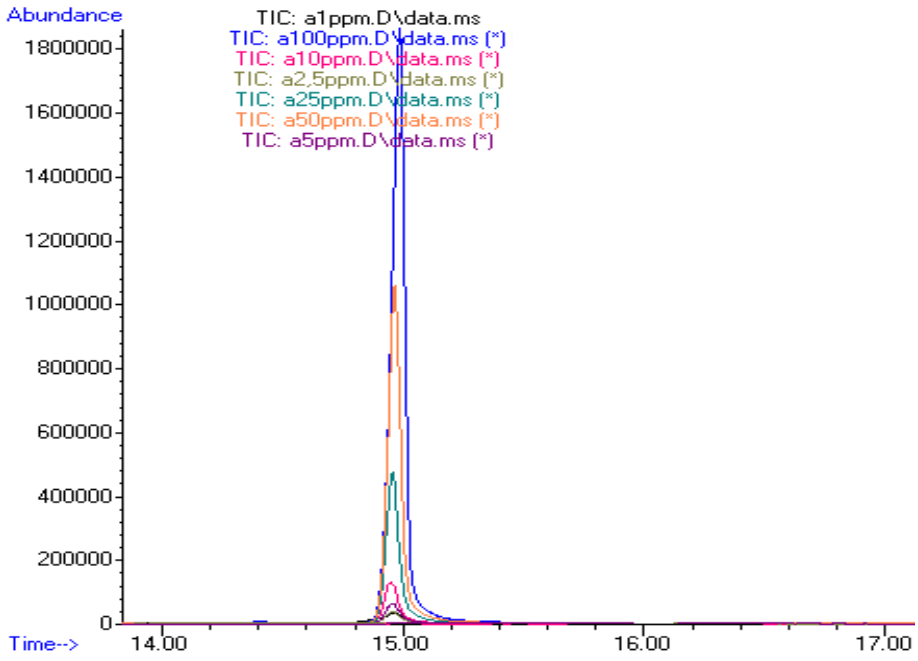

Figure 3. GC-MS chromatograms of β-sitosterol (1, 2.5, 5, 10, 25, 50 and 100 µg/mL), (HP-5 MS column (0.25 µm, 30 m × 0.25 mm I.D., initial temperature was 150 ºC, held for 2 min, increased to 220 ºC at a rate of 30 ºC /min, held for 2 min, and finally to 300 ºC at a rate of 20 ºC /min and held for 9.7 min)

3.1.2. Linearity

The linearity of β-sitosterol was studied between 1-100 µg/mL concentration range. The calibration curve was evaluated by its correlation coefficient. The calibration equation and correlation coefficient are y=184.2x-742.7 and (r = 0.9998), respectively. The standard deviations of the slope and intercept of the calibration curves were 11.31 and 24.49, respectively.

3.1. 3. Precision and accuracy

The precision of the GC-MS method was determined by repeatability (intra-day) and intermediate precision (inter-day). Repeatability was evaluated by analyzing quality control samples six times per day, at three different concentrations which were quality control samples. The intermediate precision was evaluated by analyzing the same samples once daily for three days. The relative standard deviation (RSD) of the predicted concentrations from the regression equation was taken as precision. The accuracy of this analytic method was assessed as the percentage relative error. For all the concentrations studied, intra- and inter-day relative standard deviation values were ≤4.97% and for all concentrations of β-sitosterol the relative errors were ≤4.60%. These results were given in Table 1. The intra- and inter-day precision of the quality control samples were satisfactory with RSD less than 4.97% and accuracy with relative error within ± 4.60% (should be less than 15 according to ICH guidance).

Table 1. Precision and accuracy of β-sitosterol

Added (µg/mL)	Intra-day			Inter-day		
	Found ± SD[a]	Precision % RSD[b]	Accuracy[c]	Found ± SD[a]	Precision % RSD[b]	Accuracy[c]
15	15.2 ± 0.528	3.47	1.33	14.9 ± 0.452	3.03	-0.67
50	52.3 ± 2.610	4.99	4.60	49.5 ± 2.464	4.97	-1.00
75	75.6 ± 3.214	4.25	0.80	75.7 ± 3.069	4.05	0.93

SD[a]: Standard deviation of six replicate determinations, RSDb: Relative standard deviation
Accuracy[c]: % relative error: (found-added)/added x100

3.1.4. Limit of detection (LOD) and quantification (LOQ)

The limit of detection (LOD) is the lowest amount of β-sitosterol in a sample which can be detected but not necessarily quantitated as an exact value. The limit of quantification (LOQ) is the lowest amount of β-sitosterol which can be quantitatively determined with suitable precision. The LOD and LOQ of the developed method were determined by injecting progressively low concentration of the standard solution under the chromatographic conditions. The lowest concentrations assayed where the signal/noise ratio was at least 10:1, this concentration was regarded as LOQ. The LOD was defined as a signal/noise ratio of 3:1. For β-sitosterol, the LOD and LOQ values were found 0.05 and 0.15 µg/mL, respectively.

3.1.5. Recovery

To determine the accuracy of the GC-MS method and to study the interference of formulation additives, the recovery was checked as three different concentration levels and analytical recovery experiments were performed by adding known amount of pure standard compounds to pre-analyzed sample of *A. caucasica* extract. The results are also given in Table 2. For β-sitosterol, recovery values were found between 98.2% and 99.7%. The results show that high reliability and reproducibility of the method.

Table 2. Recovery of β-sitosterol in *A. caucasica*

Alchemilla caucasica extract	Intra-day			Inter-day	
	Added (µg/mL)	Found ± SD[a]	% Recovery ± SD[a]	Found ± SD[a]	% Recovery ± SD[a]
(50 µg/mL)	10	9.98 ± 0.412	99.8 ± 4.13	9.97 ± 0.361	99.7± 3.62
	50	49.2 ± 2.432	98.4 ± 4.94	49.1 ± 1.312	98.2 ± 2.67
	100	98.7 ± 3.044	98.7 ± 3.08	99.1 ± 4.073	99.1 ± 4.11

SD[a]: Standard deviation of six replicate determinations

3.1.6. Stability

β-sitosterol samples were stable when kept at room temperature, +4 ºC and -20 ºC refrigeration temperature for 24 h and refrigerated at +4 and -20 ºC for 72 h. The results are within the acceptance range of 90-110%.

3.2. Application of Method

The developed method was applied for the determination of β-sitosterol in *A. caucasica* plant. The *A. caucasica* methanol solution was injected to GC-MS system and the area of β-sitosterol peak was measured. From the calibration curve, the amount of β-sitosterol in *A. caucasica* plant was calculated. The retention time of β-sitosterol in sample solutions was 14.9 min (Figure 4). The amount of β-sitosterol was found in *A. caucasica* methanol extract was 22.6 µg/1.0 mg with percent recovery 2.26% for β-sitosterol. Figure 4 shows the partial version of the chromatogram. There are other peaks observed from the *A. caucasica* methanol extract. Analysis time is 20 minutes. There are other peaks before 15 minutes. The phyto-constituents were identified with 13-docosenamide (Z) (59.77%), octadecenoic acid 2,3-dihydroxy-propyl ester ester (15.34%) and hexadecanoic acid 2-hydroxy-1-(hydroxymethyl)ethyl ester (7.59%). Other fatty acids and fatty acid esters identified were linoleic acid (0.69%), linolenic acid (1.96%), octa decanoic acid (0.56%) and n-hexadecanoic acid (1.81%).

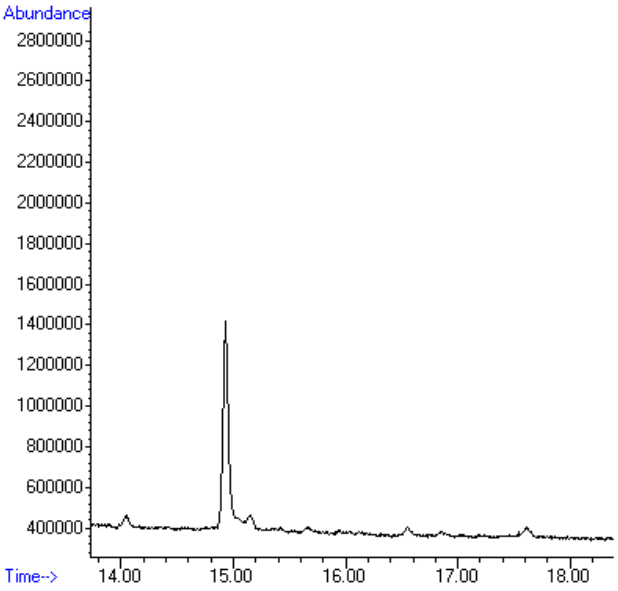

Figure 4. A typical chromatogram of *A. caucasica* (1.0 mg/mL)

4. Conclusion

In the present work, a new, simple and sensitive GC-MS method has been developed for the quantitation of β-sitosterol in *A. caucasica*. The method was validated to track the active principles in the complex mixture of herbal ingredients. The method could be extended for the marker-based standardization of other herbal products containing β-sitosterol. The method was found to be simple, precise, accurate, specific and sensitive and can be used for routine quality control of herbal raw materials and for the quantification of these compounds in plant materials.

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