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Original article

Simultaneous determination of α-amyrin and β-sitosterol in *Centranthus longiflorus* Stev. Subsp. *longiflorus* Stev and *Iris taochia* Woronow ex Grossh by GC-MS method

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Summary. Turkey is rich in terms of triterpenes and sterols flora. Triterpenes and sterols hold out for medicinal advances and new drugs. This paper describes a simple, precise, sensitive and specific method for the simultaneous determination of α-amyrin and β-sitosterol in *Centranthus longiflorus* and *Iris taochia* by gas chromatography-mass spectrometry (GC-MS) method. The retention times of α-amyrin and β-sitosterol were found 16.1 and 15.1 min, respectively. The linear ranges in this developed method were 1-100 and 5-750 μg/mL for α-amyrin and β-sitosterol, respectively. The intra- and inter-day precisions, expressed as the relative standard deviation (RSD), were less than 4.97 and 4.99%, determined from quality control samples for α-amyrin and β-sitosterol, and accuracy was within 2.00 and 4.60% in terms of relative error, respectively. The percentage recovery obtained for α-amyrin and β-sitosterol were 99.7 and 99.8%, respectively. Limit of detection and quantification for α-amyrin were 5 and 15 ng/mL, for β-sitosterol 50 and 150 ng/mL, respectively. The developed method can be used for routine quality control analysis of α-amyrin and β-sitosterol in dried whole plant of *Centranthus longiflorus* and *Iris taochia*.

Key words: cholesterol-lowering effect, phytosterol, triterpene, validation

1. Introduction

Turkey has an excellent flora and is one of the richest countries in the world in terms of plant diversity. Approximately 10,500 plant species have been recorded in this flora and the endemism rate of plants is approximately 30% (1). *Centranthus longiflorus*, which belongs to the family Valerianaceae, is known as red valerian in Turkey (2).

The plant is widely distributed in the northern, southern and central Anatolian regions of Turkey (2), endemic to the Mediterranean region (Lebanon, Syria, Turkey, Italy and Palestine). There are 3 species of Centranthus in Turkey: *Centranthus ruber, Centranthus longiflorus* and *Centranthus calcitrapa*. The aerial parts and roots of these species are mostly used for sedative, antispasmodic, anthiolitic, familial hypercholesterolemia, coronary artery disease and preventing colon cancer purposes in traditional Turkish medicines (3, 4). Some previous phytochemical studies on these species have revealed the presence of iridoids, monoterpenes and glyceridic acids (5).

It was reported in various scientifically based experiements that these plants have antioxidant and enzyme inhibitor properties. Ethanol extracts obtained from 6 species including *Centranthus longiflorus* have showed superoxide anion radical scavenging activity depending on concentration (3). It was evaluated that two plant extracts of Turkish origin, *Centranthus longiflorus* and *Cerinthe minor*, enzyme inhibitor activities, total phenol and flavonoid content, and antioxidant activities. According to the study results, the extracts

showed the highest anticholinesterase activity. It was also found that the extracts of both plants showed a high degree of radical scavenging activity (1). Ethanolic extracts and aqueous extracts from different plant parts of *Centranthus longiflorus* have showed antibacterial activity against *Staphylococcus epidermis* and ethanolic extracts have stronger antibacterial effects than aqueous extracts (2).

Iris taochia is a species in the genus Iris. *Iris taochia* lived in a mountainous area of the Black Sea to the current borders of Georgia, Armenia and Turkey. This plant is endemic to the Caucasus (6). Native to upland basalts in the north-eastern corner of Turkey around especially in Erzurum. It forms dense clumps, with grey-green leaves, simple stems of similar height, with 1-3 branches, a flowers in various shades from white, yellow to purple. They also have yellowish or white, tipped yellow beard. It is cultivated as an decorative plant in temperate zones. It is sometimes known as *Iris toochii*, or *Iris taochii*. It is commonly known as 'Autum iris' or 'Tortum süseni', 'Tortum Iris' in Turkish. Like many other irises, most parts of the plant are poisonous (rhizome and leaves), if mistakenly ingested can cause stomach pains and vomiting. Also handling the plant may cause a skin irritation or an allergic reaction (6).

Terpenes play an important role as signal compounds and growth regulators (phytohormones) of plants, accessory pigments during photosynthesis (7), and have shown antimicrobial activities (8). They include monoterpenoids, iridoids, sesquiterpenoids, sesquiterpene lactones, diterpenoids, triterpenoid saponins, steroid saponins, cardenolides and bufadienolides, phytosterols, cucurbitacins, nortriterpenoids, other triterpenoids, and carotenoids (7). Triterpene saponins are the most val ued in terms of pharmacology. They have anti- inflammatory, hypoglycemic and most important anticancer activity (9).

Sterols are important constituents of all eukaryotes and play vital role in plant cell membranes. Plant sterols possess valuable physiological activities, they are biogenetic precursors of many hormones and oviposition stimulants of some insects (10).

Among all phytochemicals, β-sitosterol is a main phytosterol found in many plants. It has been reported to show anti-inflammatory, antineoplastic, antipyretic, immunomodulating, antioxidative and antiviral activity (11, 21). Also triterpenoids are among commonly present secondary metabolites in plants. α-amyrin, a pentacyclic triterpenoid, has been reported to show anti-inflammatory and antioxidant properties (12, 22).

Triterpenes and sterols certainly give a much needed hope for medicinal advances and new drugs. The development of science and technological lows for even better opportunities for separation and isolation of these substances. Extracts are normally mixtures of many compounds having different polarities. Consequently, the purification step is very important (13).

However, no method was applied for quantifying the presence of α-amyrin and β-sitosterol simultaneously from *Centranthus longiflorus* and *Iris taochia*. Therefore, analytical methods for their separation and simultaneous quantification are required for quality control purpose. A survey of literature reveals that no GC-MS method for simultaneous determination of these two compounds. Hence, in present research work, a simple, rapid, precise and accurate GC-MS method has been developed and validated using International Conference on Harmonization guidelines (14) for simultaneous determination and quantification of α-amyrin and β-sitosterol form dried whole plant powder of *Centranthus longiflorus* and *Iris taochia*.

2. Materials and Methods

2.1. Plant materials

Centranthus longiflorus and *Iris taochia plants* were collected from Tortum in the province of Erzurum in April and June 2016 and authenticated by Davis (15) and Richardson (16).

2.2. Extraction of Centranthus longiflorus and Iris taochia

The drying process of the plants and all the remaining studies were carried out in Atatürk University Science Faculty Genetics Laboratory. Ethanol extracts were prepared from the dried whole plant of *Centranthus longiflorus* and *Iris taochia*. Extractions of ethanol from the plants were performed according to Kotan *et al.* (17). In this method, ethanol is added in a volume of about 2 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 ethanol is removed. After all of the ethanol is removed, the whole dry extracts are scraped with a spatula and weighed. These are stored at + 4°C until use in study.

2.3. GC-MS conditions

Chromatographic analysis was carried out on an Agilent 7820A gas chromatography system equipped with 5977 series mass selective detector, 7673 series autosampler and Agilent chemstation (Agilent Technologies, Palo Alto, CA). HP-5 MS column with 0.25 μm film thickness (30 m × 0.25 mm I.D., USA) was used for separation. Splitless injection was used and the carrier gas was helium at a flow rate of 1.5 mL/min. The injector volume was 1 mL. The MS detector parameters were transfer line temperature 280 C, solvent delay 3 min and electron energy 70 eV.

The GC temperature gradient program was as follows: initial temperature was 150°C, held for 2 min, increased to 220°C at a rate of 30°C/min, held for 2 min, and finally to 300°C at a rate of 20°C/min and held for 9.7 min. The MS detector parameters were: transfer line temperature 280°C; solvent delay 3 min; electron energy 70 eV; the MS was run in electron impact mode with selected ion monitoring (SIM) for quantitative analysis [m/z 145 for β-sitosterol, m/z 218 for α-amyrin].

2.4. Standards and quality control samples

Reference standards of both α-amyrin and β-sitosterol were obtained from Sigma (St. Louis, MO, USA). Stock solutions of α-amyrin and β-sitosterol were prepared by dissolving the accurately weighed reference compounds in chloroform to give a final concentration of 1000 μg/mL of both. The solution was then serially diluted with chloroform to achieve standard working solutions at concentrations of 1, 2.5, 5, 10, 25, 50, 100 μg/mL and 5, 10, 25, 50, 100, 250, 500, 750 μg/mL for α-amyrin and β-sitosterol, respectively. Structural formula of α-amyrin and β-sitosterol are shown in Figure 1. All solutions were stored at 4C 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 α-amyrin and 15, 150 and 600 μg/mL for β-sitosterol.

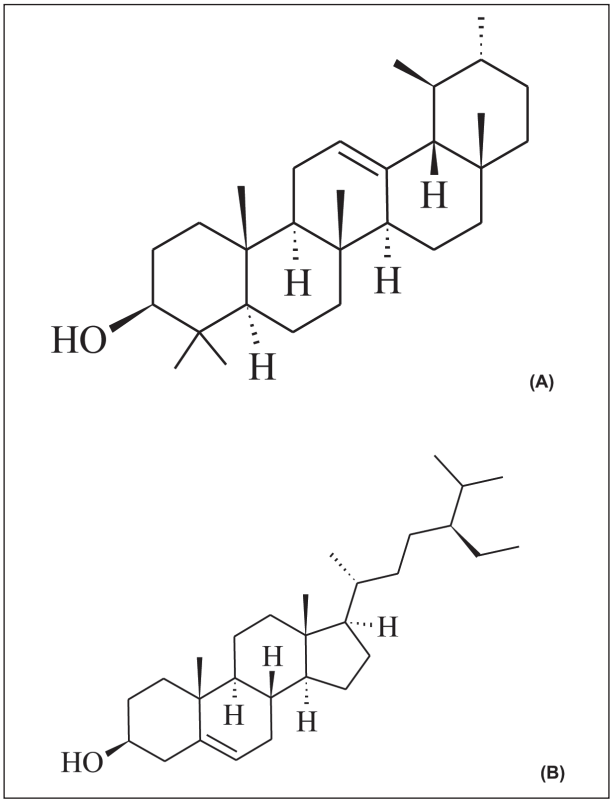

Figure 1. Structural formula of α-amyrin (A) and β-sitosterol (B)

3. Results and Discussion

3.1. Validation of method

To evaluate the validation of the present method, parameters as specificity, linearity, precision, accuracy, limit of detection (LOD), limit of quantification (LOQ) and recovery were investigated according to ICH validation guidelines (14).

3.2. Selectivity

The selectivity of the GC-MS method was investigated by observing interferences between α-amyrin and β-sitosterol. For GC-MS, electron impact mode with selected ion monitoring (SIM) was used for quantitative analysis [m/z 145 for β-sitosterol and m/z 218 for α-amyrin]. The mass spectra of the amyrin

and β-sitosterol are shown in Figure 2. The retention times of α-amyrin and β-sitosterol in GC-MS method was approximately 16.1 and 15.1 min with good peak shape (Figure 3, 4).

3.3. Linearity

Linearity was determined for α-amyrin in the range of 1-100 µg/ml and for β-sitosterol 5-750 µg/mL The calibration curves constructed were evaluated by their correlation coefficients. The calibration equations from three replicate experiments demonstrated the linearity of the method. Standard deviations of the slope and intercept for the calibration curves were given in Table 1.

3.4. 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 £4.99% and

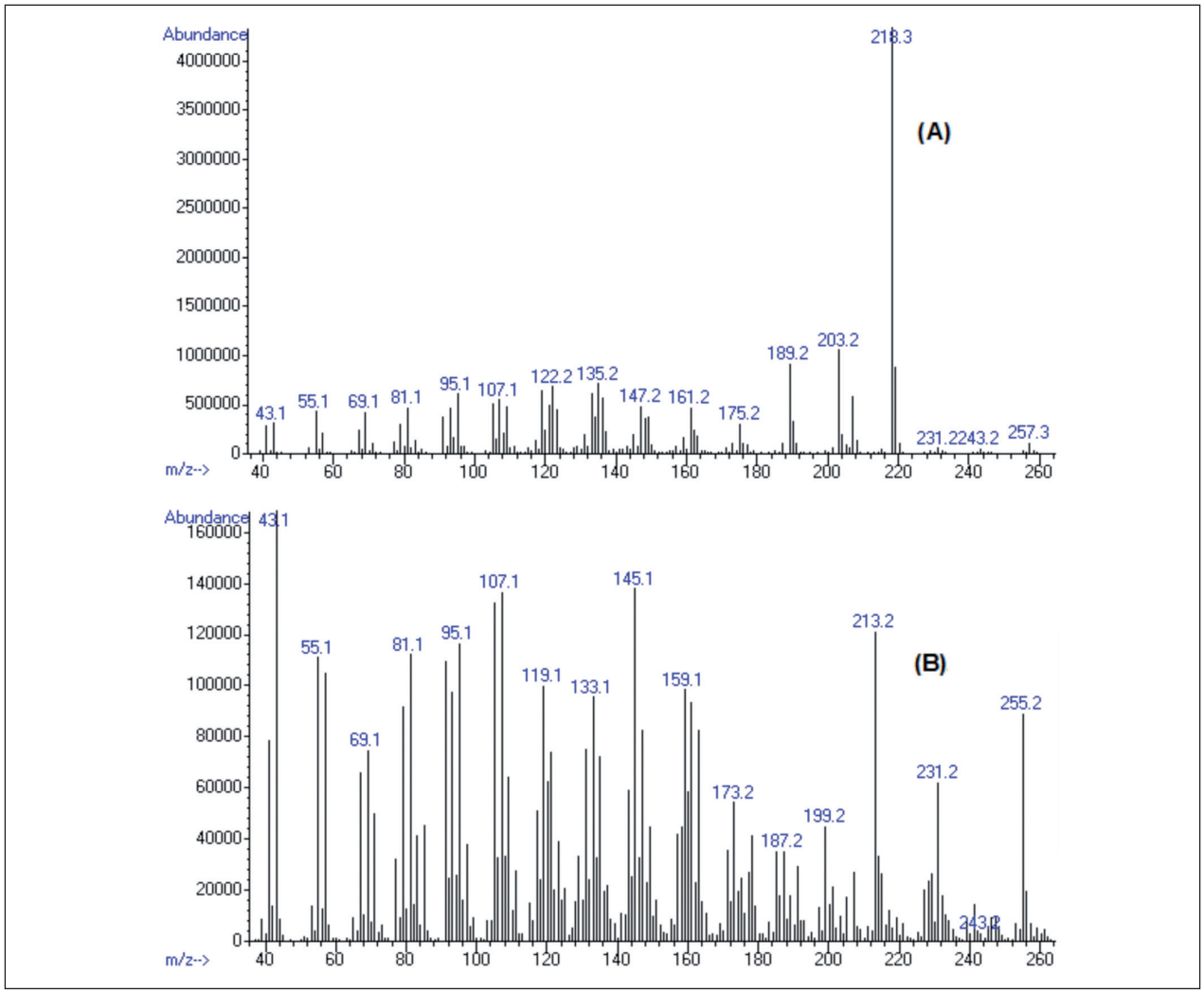

Figure 2. MS spectra of α-amyrin (A) and β-sitosterol (B)

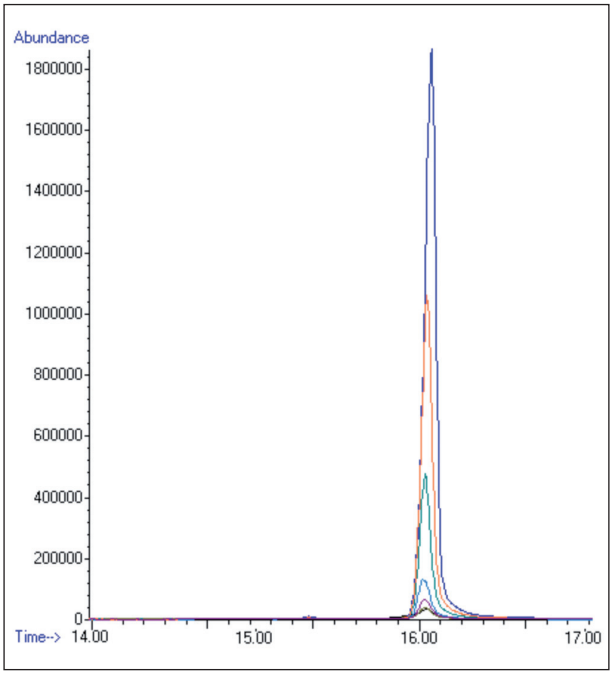

Figure 3. GC-MS chromatograms of α-amyrin (1, 2.5, 5, 10, 25, 50 and 100 μg/mL)

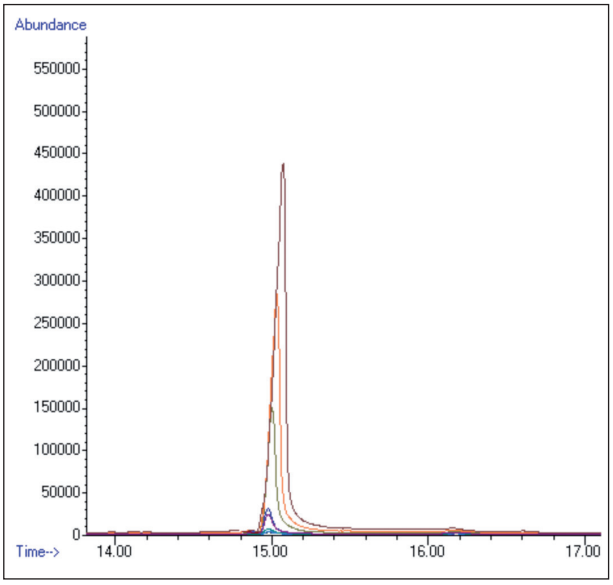

Figure 4. GC-MS chromatograms of β-sitosterol (5, 10, 25, 50, 100, 250, 500 and 750 μg/mL)

for all concentrations of α-amyrin and β-sitosterol the relative errors were £4.60%. These results were given in Table 2.

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

The limit of detection (LOD) is the lowest amount of α-amyrin and β-sitosterolin a sample which can be detected but not necessarily quantitated as an exact value. The limit of quantification (LOQ) is the lowest amount of α-amyrin and 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. The results are shown in Table 1.

Table 1. Features of the calibration curves of α-amyrin and β-sitosterol

Parameters	**α-amyrin**	**β-sitosterol**
Linearity (μg/mL)	1-100	5-750
Regression equation[a]	y=19090x-7645.1	y=582.39x-7668.6
Standard deviation of slope	113.85	7.023
Standard deviation of intercept	94.39	241.64
Correlation coefficent	0.9938	0.9967
Standard deviation of correlation coefficent	$5.0x10^{-3}$	$2.9x0^{-3}$
Limit of detection (ng/mL)	5	50
Limit of quantitation (ng/mL)	15	150

[a]*Based on three calibration curves, y: peak-height, x: α-amyrin and β-sitosterol concentration*

Table 2. Precision and accuracy of α-amyrin and β-sitosterol

Added (µg/mL)	Found ± SD[a]	Intra-day Precision % RSD[b]	Accuracy[c]	Found ± SD[a]	Inter-day Precision % RSD[b]	Accuracy[c]
α-amyrin						
15	14.9 ± 0.452	3.03	-0.67	15.2 ± 0.528	3.49	1.33
50	49.5 ± 2.464	4.97	-1.00	52.3 ± 2.610	4.99	4.60
75	75.7 ± 3.069	4.05	0.93	75.6 ± 3.214	4.25	0.80
β-sitosterol						
15	15.3 ± 0.318	2.08	2.00	15.2 ± 0.427	2.81	1.33
150	148.7 ± 5.074	3.41	-0.87	146.8 ± 5.216	3.55	-2.13
600	597.4 ± 13.47	2.25	-0.43	596.1 ± 22.08	3.70	-0.65

SD[a]: Standard deviation of six replicate determinations
RSD[b]: Relative standard derivation
Accuracy[c]: % relative error: (found-added)/addedx100

3.6. Recovery

To determine the accuracy of the GC-MS methods 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 samples of *Centranthus longiflorus* and *Iris taochia* extracts. These values are also listed in Table 3.

3.7. Application of method

The developed GC-MS method was used for simultaneous determination of α-amyrin and β-sitosterol from whole plant powder of *Centranthus longiflorus* and *Iris taochia*. The sample working solution (1 µL) was injected and the height of both α-amyrin and β-sitosterol peak was measured. From the calibration curve, the amount of α-amyrin and β-sitosterol in dry powder of *Centranthus longiflorus* and *Iris taochia* was calculated. The retention time of α-amyrin and β-sitosterol in sample solutions were 16.1 and 15.1 and in the standard solution was found to be 16.1 and 15.1 respectively. The mean amounts of α-amyrin and β-sitosterol found in whole plant powder of *Centranthus longiflorus* were 6.408 µg/10 mg and 226.603 µg/10 mg respectively with percent recoveries 0.064% and 2.26% for α-amyrin and β-sitosterol, respectively. The mean amounts of α-amyrin and β-sitosterol found

Table 3. Recovery of α-amyrin and β-sitosterol in *Centranthus longiflorus* and *Iris taochia*

Plant	Added (µg/mL)	α-amyrin Found ± S.D.[a]	α-amyrin % Recovery % RSD[b]	β-sitosterol Found ± S.D.[a]	β-sitosterol % Recovery % RSD[b]
C. longiflorus					
	10	9.97 ± 0.361	99.7 (3.62)	9.98 ± 0.412	99.8 (3.62)
(20 µg/mL)	50	49.1 ± 1.312	98.2 (2.67)	49.2 ± 2.432	98.4 (2.67)
	100	98.7 ± 3.044	98.7 (3.08	99.1 ± 4.073	99.1 (3.08
I. taochia					
	10	9.96 ± 0.426	99.6 (4.27)	9.95 ± 0.397	99.5 (3.99)
(20 µg/mL)	50	50.2 ± 1.492	100.4 (2.97)	50.3 ± 1.986	100.4 (3.95)
	100	99.4 ± 3.471	99.4 (3.49)	100.2 ± 3.436	100.2 (3.43)

SD[a]: Standard deviation of six replicate determinations
RSD[b]: Relative standard derivation

Table 4. Application of α-amyrin and β-sitosterol in *Centranthus longiflorus* and *Iris taochia* (10 mg/mL)

Plant	α-amyrin		β-sitosterol	
	Found ± SD[a] (µg/mL)	Content %	Found ± SD[a] (µg/mL)	Content %
C. longiflorus	6.408 ± 0.261	0.064	226.603 ± 9.412	2.26
I. taochia	4.598 ± 0.126	0.045	199.889 ± 7.397	1.99

SD[a]: Standard deviation of six replicate determinations

in whole plant powder of *Iris taochia* were 4.598 µg/10 mg and 199.889 µg/10 mg respectively with percent recoveries 0.045% and 1.99% for α-amyrin and β-sitosterol, respectively (Table 4).

3.8. Comparison of methods

During GC-MS analysis, several different oven temperatures were tried for separation of α-amyrin and β-sitosterol from other phytochemicals present in whole plant powder of *Centranthus longiflorus* and *Iris taochia*. Good separation was achieved with the oven temperature: initial temperature was 150 C, held for 2 min, increased to 220 C at a rate of 30 C/min, held for 2 min, and finally to 300 C at a rate of 20 C/min and held for 9.7 min. The retention time values for α-amyrin and β-sitosterol were 16.1 and 15.1, respectively. Figure 5 and Figure 6 show typical GC-MS chromatogram of standard α-amyrin and β-sitosterol in extracts of dried whole plant powder of *Centranthus longiflorus* and dried whole plant powder of *Iris taochia*.

Literature survey revealed that some of the related methods were reviewed. A qualitative normal phase high performance thin layer chromatography (HPTLC) method was reported (18) for separating and determining α-amyrin and lupeol from *Brassica oleracea* L. leaf extracts. HPTLC silica gel 60F254 plates were used as stationary phase with the mobile phase as n-hexane-ethyl acetate in a volume ratio of 5:1. Post derivatization was carried out using anisaldehyde-sulphuric acid reagent. Identification of α-amyrin and lupeol was carried out by visual comparison of the colour of α-amyrin and lupeol after derivatization. The reported method was unable to resolve the isomeric

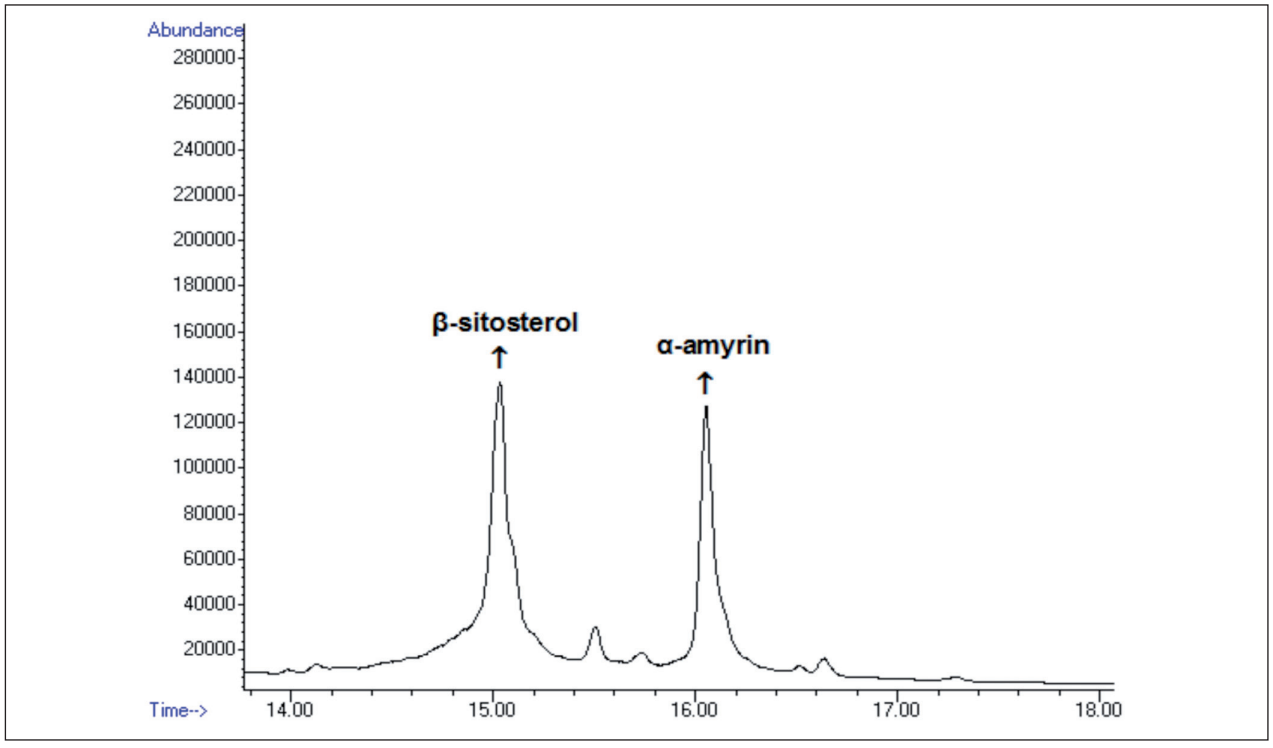

Figure 5. A typical chromatogram of *Centranthus longiflorus (*10 mg/mL)

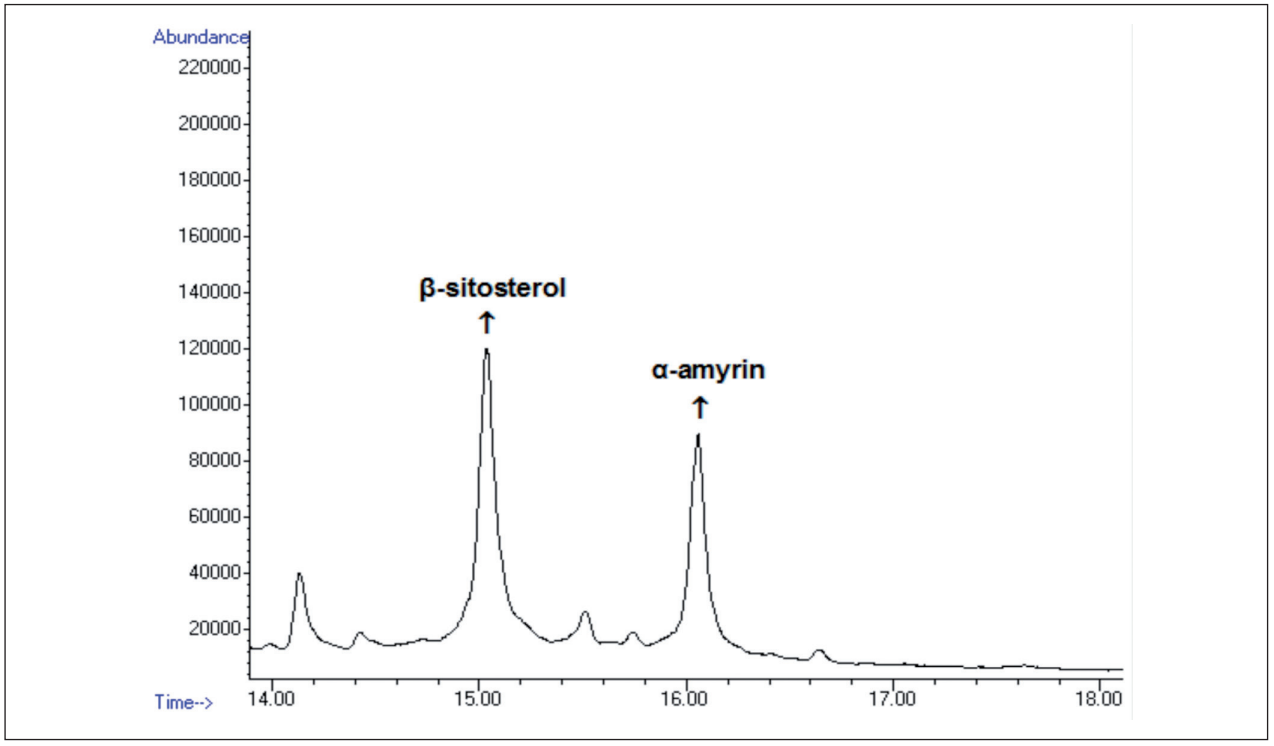

Figure 6. A typical chromatogram of *Iris taochia (*10 mg/mL)

compounds α-amyrin and lupeol, because there was no significantdifference in their R_f values.

In a reported method (12) 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*. Solid phase extraction was used to remove the matrix (19).

A GC-MS method was reported for analysing compounds in *Salvia bicolour* Desf. extract. β-sitosterol and lupeol were detected. The retention time observed was 41.04 mins for β-sitosterol and 41.5 mins for lupeol (20). Both methods were time consuming. Therefore, in the present research work, in order to standardize the plants with these markers, a precise and accurate GC-MS method for simultaneous estimation of α-amyrin and β-sitosterol from the extract of whole plant powder of *Centranthus longiflorus* and *Iris taochia* was developed. Also, α-amyrin and β-sitosterol have also been quantified.

In conclusion, in the present work, a new, simple and sensitive GC-MS method has been developed for the quantitation of α-amyrin and β-sitosterol in whole plant powder of *Centranthus longiflorus* and *Iris taochia*. 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 product containing α-amyrin and 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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