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Identification of steroid hormones in pomegranate (*Punica granatum*) using HPLC and GC–mass spectrometry

Don Woong Choi [a,*], Jee Yeun Kim [a], Seon Hee Choi [a], Hee Su Jung [a], Hyo Joo Kim [b], So Yean Cho [c], Chan Soon Kang [a], Seung Yeup Chang [c]

[a] *Office of Analysis Evaluation, Korea Food and Drug Administration (KFDA), Eunpyung-Gu, Seoul 122-704, Korea*
[b] *Center of Quality Control and Assurance, Ilhwa Corp., 1 Sootaek-Dong, Guri City, Gyunggi Province 471-711, Korea*
[c] *Division of Natural Medicines Standardization, Korea Food and Drug Administration (KFDA), Seoul 122-704, Korea*

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Abstract

Although it has been known that pomegranate (*Punica granatum* L.) contains several steroid hormones, concrete experimental proofs about that have not been published until now. In order to identify and evaluate the contents of steroid hormones including estrone in pomegranate, we analyzed pomegranate seed, fruit juice and commercial preparations. We developed a reproducible and sensitive method for separation and identification of steroid hormones in pomegranate samples using both high performance liquid chromatography (HPLC)–PDA and gas chromatography (GC)–MS. In case of HPLC, an isocratic elution method using 35% aqueous acetonitrile solution at 1.0 ml/min with photodiode-array (PDA) detection at 225 nm and 254 nm was found to optimally separate and identify the steroid hormones from the pomegranate samples with a run time of less than 30 min. The pomegranate samples were comparatively analyzed to the HPLC results by GC/FID or GC/MS detection on a HP-1 (30 m length, 0.32 mm I.D.) with helium as carrier gas under the oven temperature control as follows: start 220 °C for 5 min, raising 5 °C per min, final 280 °C for 10 min. The HPLC and GC methods were successfully applied to the identification of steroid hormones in pomegranate samples. Our results suggested that there were no steroid estrogens including estrone, estradiol and testosterone in pomegranate seed, fruit juice and preparations. Consequently, we assumed that the previously reported analysts of pomegranate were misunderstood their analytical results according to either the estrogen-like effects or similarity of peak retention time and Rf values in experiments.

Keywords: Pomegranate; Steroid estrogens; Estrone; Testosterone; HPLC–PDA; GC/MS

1. Introduction

Over the last decades, there has been a dramatic increase in interest on phytoestrogens, a member of classes of polyphenolic substances synthesized by plants and exerting a estrogen-like effect, mainly associated with the cure of specific diseases or possible adverse toxicity to the reproductive organs by endocrine disrupting potential (Gutendorf & Westendorf, 2001; Wang, Prasain, & Barnes, 2002). The pomegranate (*Punica granatum*, Punicaceae) has been known to considerable pharmacological properties with anti-microbial, anti-viral, anticancer, potent anti-oxidant and anti-mutagenic effects (Negi, Jayaprakasha, & Jena, 2003), and been used in the markets in the preparations of tinctures, juice, cosmetics and therapeutic formulae (Kim, Mehta, Yu, & Lansky, 2002). It has been recently reported that the

* Corresponding author. Tel.: +82 2 380 1692/93; fax: +82 2 380 1625.
E-mail addresses: cdwkje@hanmail.net, cdwkje@kfda.go.kr (D.W. Choi).

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pomegranate peels and seed oils contains a substantial amount of polyphenols such as sugar-bound flavonoids quercetin and kaempferol (Chauhan & Chauhan, 2001), flavonoid diglycosid (El-Toumy & Rauwald, 2002), ellagic acid and ellagic tannin (Poyrazoglu, Gokmen, & Aruk, 2002) and organic acids (Heftmann, Ko, & Bennett, 1966). On the other hand, there were some reports that the pomegranate seeds and peel extracts contain steroid hormones including estrone (Dean, Exlby, & Goodwin, 1971; Moneam, el Sharaky, & Badreldin, 1988), estradiol (Abd El Wahab, El Fiki, Mostafa, & Hassan, 1998) and testosterone (Lau, Holmes, & Woo, 2003). Most of these researches have been focused on estrogen-like activities through in vivo animal tests and in vitro cytokines studies or on the limited classical analysis techniques. There were many analytical methods for the identification and determination of steroid hormones from pomegranate extracts. In the case of quantitative measurements of estrone from pomegranate seeds, much of work on the identification and determination of steroid estrogens has been done either by the same Rf value in thin layer chromatography (TLC) (Moneam et al., 1988) or by the same peak retention time in high performance liquid chromatography (HPLC) associated with UV detector (Dean et al., 1971). These previous experiments, however, might have the risk of mistake owing to overlooking the analytical limitations of their approaches.

The adulterations with synthetic chemicals, on the other hand, were common problems with herbal medicines and these could potentially cause social problems including adverse effects. The adulterations with steroids or sildenafil derivatives have been especially significant problems in the East Asian countries (Huang, Wen, & Hsiao, 1997). Because of the social consequences and life threatening complications, the adulterated food preparations might be serious obstacles to the authorities doing management of food safety.

The objective of the present study, therefore, was to identify estrone, estradiol and testosterone [Fig. 1] present in pomegranate itself and pomegranate preparations. The analytical measurements of steroid estrogens and testosterone were performed using HPLC with photodiode-array (PDA) detection. And also, comparative analytical techniques including gas chromatography (GC)-flame ionization detection (FID) and GC-mass selective detection (MSD) were simultaneously applicated for the purpose of the reconfirmation of the HPLC results. These methods combined all the desired features for a rapid, sensitive, and reproducible for the identification of steroid hormones in pomegranate samples.

2. Materials and methods

2.1. Chemicals

Estrone, estradiol and testosterone were purchased from Sigma Chemical (St. Louis, MO, USA). Methanol, acetonitrile and water were of HPLC grade and obtained from Merck (Darmstadt, Germany). Hydrochlo-

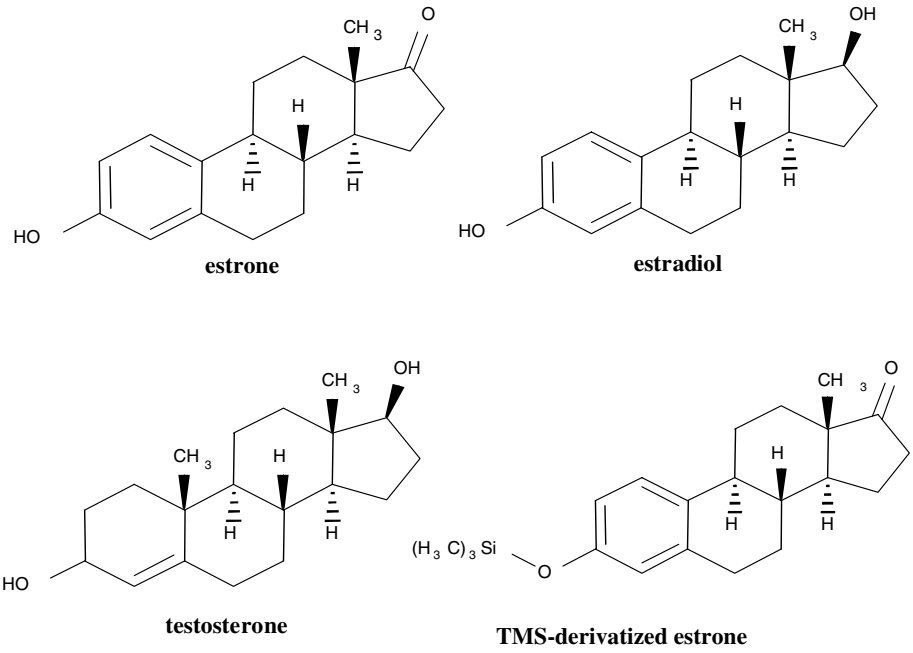

Fig. 1. Molecular structures of the estrone, estradiol, testosterone and TMS-derivatized estrone compound used for HPLC and GC/MS method: 1, estrone; 2, estradiol; 3, testosterone; 4, TMS-derivatized estrone.

ric acid, acetic acid and nitric acid, anhydrous sodium sulfate were purchased from Wako Pure Chemical Industries, Ltd. (Japan).

2.2. Pomegranates

The pomegranate ripen fruits cultivated in Iran were purchased from the local market, Lotte Department Store (Seoul, Korea). The pomegranate fruits cultivated in Korea were obtained from the Medicinal Plant Research Center (Korea Food and Drug Administration, Seoul, Korea).

2.3. Instruments

HPLC analysis was carried out using a Waters Alliance® system (Milford, MA, USA) consisting of pump control separation module (Model 2690), a programmable photodiode array detector (Model 996), an autosampler (Model 717). Samples were introduced via autoinjector with a 20 μl and all chromatographic separations were carried out at ambient temperature. Data acquisition and analysis were performed on a computer using the Millenium 32® chromatography software (Waters Assoc.), which communicated with the HPLC equipment. GC/FID and GC/MS were applicated with using a HP 6890 Series® (Hewlett Packard, USA), HP 5972 mass selective detector (Hewlett Packard, USA) and Polaris Q GC/MS® system (Thermo Finigan, UK). Ultra-centrifuge 5417 (Eppendorf, USA) was used for the analyses. All eluents were filtered through 0.45 μl filters (Millipore) on use.

2.4. Preparations of pomegranate extracts

Approximately 7 kg of pomegranate maturing fruits were obtained from local markets and the Medicinal Plant Research Center (KFDA), respectively. First of all, for the preparation of pomegranate fruit juice sample, the pomegranate fruits were sliced to pieces, grinded to juice. After adding 10 ml of water to 10 g of the juice in 250 ml of Erlenmeyer flask, it was extracted three times with 10 ml of methanol, separately. The aqueous methanol extracts were gathered and concentrated with evaporator. The residue was dissolved with 1 ml of methanol. Secondly, for the preparing of pomegranate seed sample, following peeling out, the strong fibrous skins covering seeds were removed. The remaining pomegranate seeds were dried under a shady spot and 2 g of the seeds were powdered with mortar and extracted with 10 ml of methanol. Each extraction procedures were repeated five times, the methanol layers were filtered and concentrated with evaporator. The residue was dissolved with 1 ml of methanol. Lastly, for the preparing of pomegranate preparation sample, 10 g of concentrated pomegranate extracts was diluted with 9 ml of water and alkalified to pH 8–9 with 1 ml of 0.1 M NaOH solution. A C_{18} solid-phase extracting cartridge was conditioned by eluting 3 ml of methanol, 6 ml of water and 6 ml of air sequentially. The diluted tincture sample was eluted through a pre-conditioned C_{18} cartridge and eluate was collected. The cartridge was washed twice with 2 ml of water, then the overall eluate was gathered and concentrated with evaporator. The residue was dissolved with 1 ml of methanol. All of the pomegranate samples were filtered through a membrane filter (pore size 0.45 μm) before instrumental measurements.

2.5. Analytical HPLC coupled with photodiode array detection (HPLC–PDA)

Analytical HPLC was conducted on a Waters Alliance® liquid chromatography with a Capcellpak® C_{18}, reverse-phase column (5 μm, 25 cm × 4 mm ID, Shiseido, Japan). For the separation of individual substances in methanolic extracts of pomegranate samples, a variety of compositions of mobile phase were studied. As the result, 35% aqueous acetonitrile was showed good resolution and response between steroid hormones and interfering constituents. The mobile phase was filtered through a 0.45 μm membrane filter and degassed by sonication prior to use. The flow rate of the mobile phase was 1.0 ml/min and the injection volume was 20 μl. All the steroid hormones have in common the cyclopentanophenanthrene nucleus (Lopez de Alda & Damia Barcelo, 2000). The UV spectrum characteristic of the estrogens presented two maxima, one at approximately 210 nm and another at 280 nm, the testosterone, on the other hand, showed a characteristic absorption maximum at about 254 nm. Therefore, the column eluate was monitored with a photodiode array detector at 225 nm for estrone and estradiol, 254 nm for testosterone, respectively. The identification of steroid hormone in the eluate was determined by peak of same retention time (min) and accordance of PDA spectrum (λ) to the standard. The amount of steroid hormones in the pomegranate samples were determined using calibration curves generated with authentic standards in the range of 0.001–0.01 mg/ml. The study of each pomegranate samples was carried out several, at least three times. Data acquisition and process were performed using software of Millenium 2000 (Waters®).

2.6. GC coupled with FID detection

The GC analysis was performed on a Hewlett-Packard 6890 mainbody and flame ionization detector (FID). The conditions of GC/FID were achieved using a HP-1 capillary column (30 m × 0.25 mm ID, 0.25 μm film thickness, Hewlett Packard Co.) and helium used as carrier gas with a linear velocity of 50 ml/min. The oven temperature program was as follows: initial tem-

perature 150 °C for 5 min, raising 10 °C per min, final temperature 280 °C for 10 min. The injector temperature was 270 °C and the detector temperature was held at 290 °C. The splitless mode was used for injection, with a purge time of 3 min. The study of each pomegranate samples was carried out several, at least three times. Data acquisition and process were performed using software of HP Chemstation (HP).

2.7. GC associated with mass detection

The GC/MS analysis to identify estrone from pomegranate samples was performed by two stages, especially. Firstly, the pomegranate samples were directly analyzed by HP 5972 mass selective detector (MSD) coupled with a HP 6890 GC. The GC/MSD was operated under the following conditions: autoinjection, splitless mode injection; injection temp. 270 °C; oven temp. programmed with initial temp. 180 °C (hold 1 min), rate 10 °C/min, final temp. 280 °C (hold 5 min); detector temp. 290 °C; run time 20 min; start time 5.0 min; damping gas flow 0.3 ml/min; electron energy 70 eV; scan range 50–650 amu; scan—TIC. From the previous GC/MS results, the Thermo Finigan Polaris Q mass spectrometer interfaced with a Trace GC 2000 (Finigan, UK) was applied secondary for identification and recon-

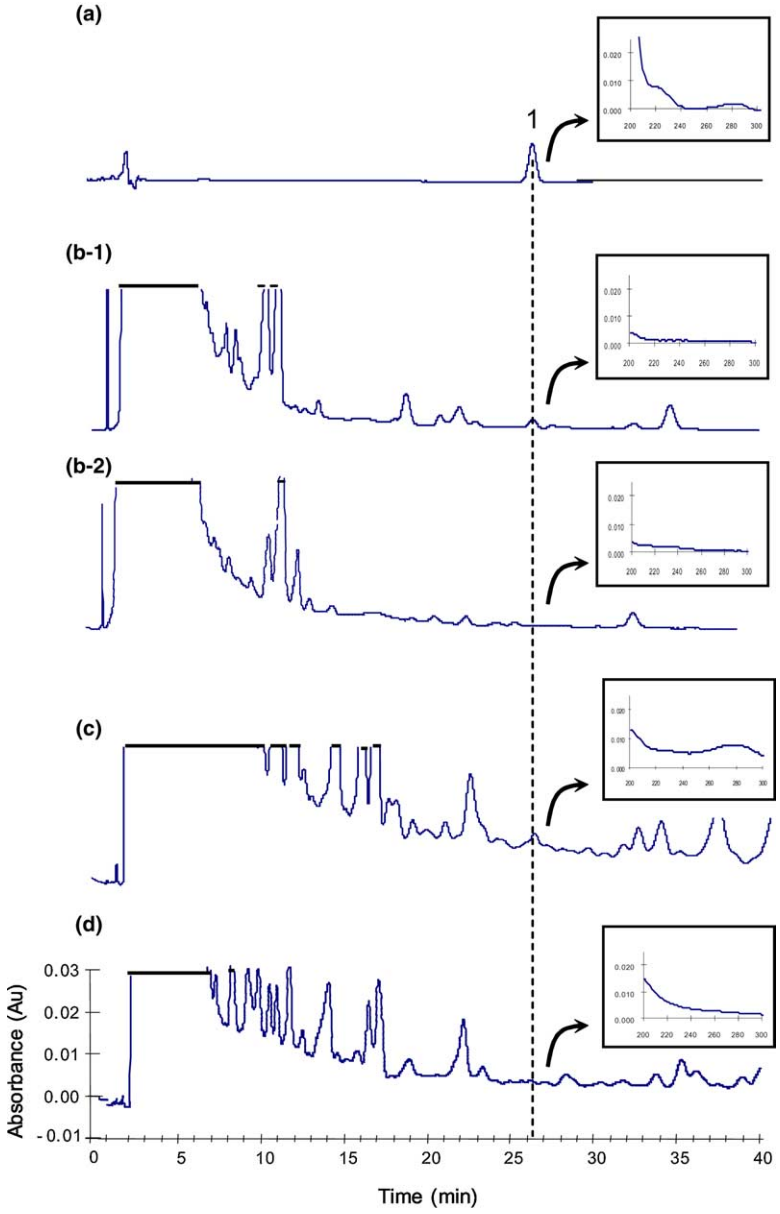

Fig. 2. Typical HPLC chromatograms and PDA spectrum obtained from estrone standard and pomegranate samples. Concentration levels of standard and sample solutions were as follows: (a) standards (1, estrone) 0.001 mg/ml; (b-1) pomegranate seed (cultivated in Iran) 1.0 g/ml; (b-2) pomegranate seed (cultivated in Korea) 1.0 g/ml; (c) pomegranate fruit juice 10.0 g/ml; (d) pomegranate preparation 10 g/ml. The peak of interest was shown by both dotted line and its PDA spectrum absorbing range from 200 nm to 300 nm.

firmation of estrone from pomegranate seed samples. For the detection and separation of a suspicious infinitesimal quantity of estrone, the analysis was performed using derivatization with trimethylsilylation according to Haber et al. (2001) before injection. Shortly described, 1 ml of water was added to 1 ml of extracted sample solution, then 100 μl of 1 M NaOH solution was added to adjust about pH 9–10 and vortexed for 30 s and equilibrated for 1 h at room temperature. Extraction was performed by adding 5 ml of chloroform, and shaking for 20 min and centrifuged for 5 min at 3000 rpm. The organic layer was separated and evaporated to dryness under nitrogen gas at 30 °C. And then, 100 μl of freshly prepared derivative solution composed of *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide (MSTFA): ammonium iodide (NH_4I): dithioerythritol (DTE) (1000:4:5, v/w/w) was added to the dry residue at 60 °C for 20 min. Estrone (10 ppm) was spiked into the pomegranate seed sample before GC/MS analysis. Those of 2 μl of derivatized sample and standard solutions, 2 μl of spiked solution were injected into GC/MS, respectively. For selected ion identification, SIM mass spectral data for each of the derivatized compounds were collected in two SIM group, namely 325 and 343 *m*/*z*. The GC/MS was tuned before each injection using perfluorotributylamine (PFTB) as tuning standard. The specificity of the GC identifications was evaluated with the accordance of peak retention time, and additionally with the total ion chromatogram (TIC) in GC/MS analysis. The estrone was identified

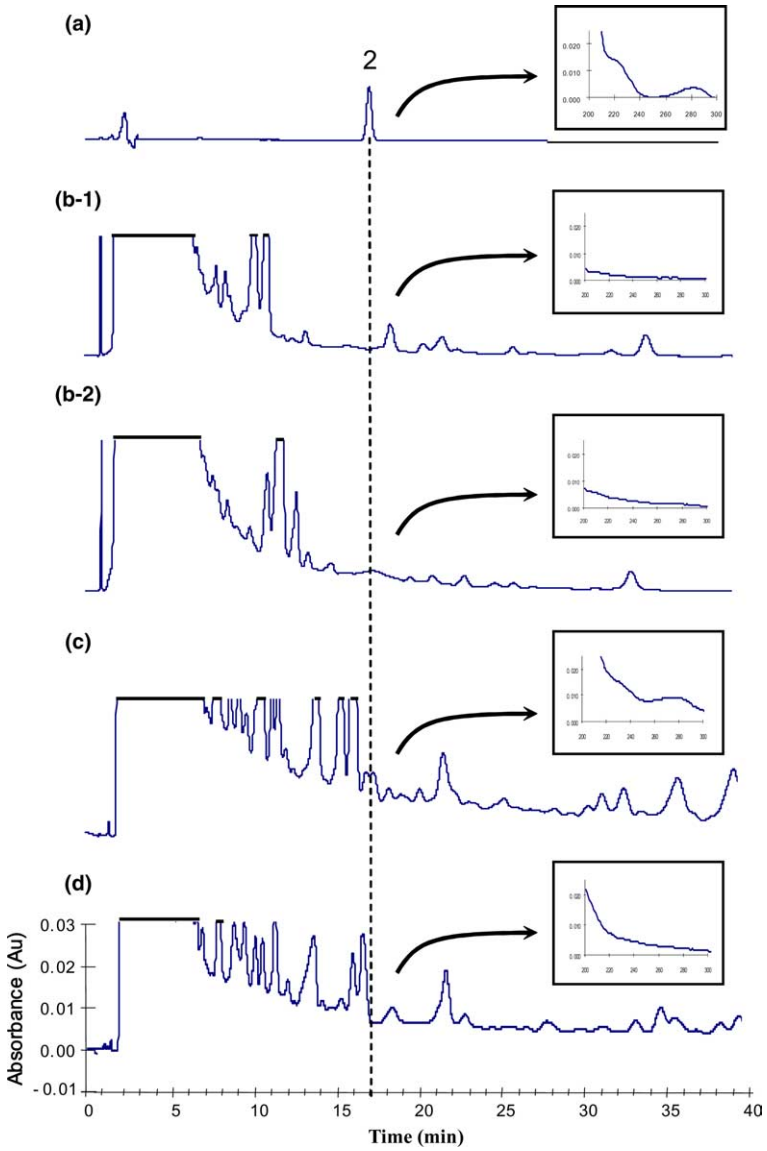

Fig. 3. Typical HPLC chromatograms and PDA spectrum obtained from estradiol standard and pomegranate samples. Concentration levels of standard and sample solutions were as follows: (a) standards (2, estradiol) 0.005 mg/ml; (b-1) pomegranate seed (cultivated in Iran) 1.0 g/ml; (b-2) pomegranate seed (cultivated in Korea) 1.0 g/ml; (c) pomegranate fruit juice 10.0 g/ml; (d) pomegranate preparation 10 g/ml. The peak of interest was shown by both dotted line and its PDA spectrum absorbing range from 200 nm to 300 nm.

by its retention time and by mass spectra comparing with it both using standard and a commercial library Wiley® mass spectral database.

3. Results and discussion

3.1. HPLC–PDA analysis

When a sample was suspected to contain a steroid hormones, the HPLC analysis provided a first indication about the presence of a steroid compounds, then the comparison of spectrums by diode-array detector allowed the determinative confirmation on the peaks with same retention time between HPLC chromatograms. The photodiode-array detector enabled the evaluation of the peak purity factors, which are very useful in the analysis of a unknown constituent by confirming both peak purity and peak identification, after a comparison of the values obtained with those of authentic standards. Preliminary studies were carried out with acetonitrile–water at various volume ratios as mobile phase, but it was very difficult to separate interference peaks because of overlap of chromatographic peaks of pomegranate sample solutions. In the subsequent studies, effects of acetonitrile amount on separation of steroid hormones were evaluated in terms of peak resolution. When using 35% acetonitrile as mobile phase, all peaks were well

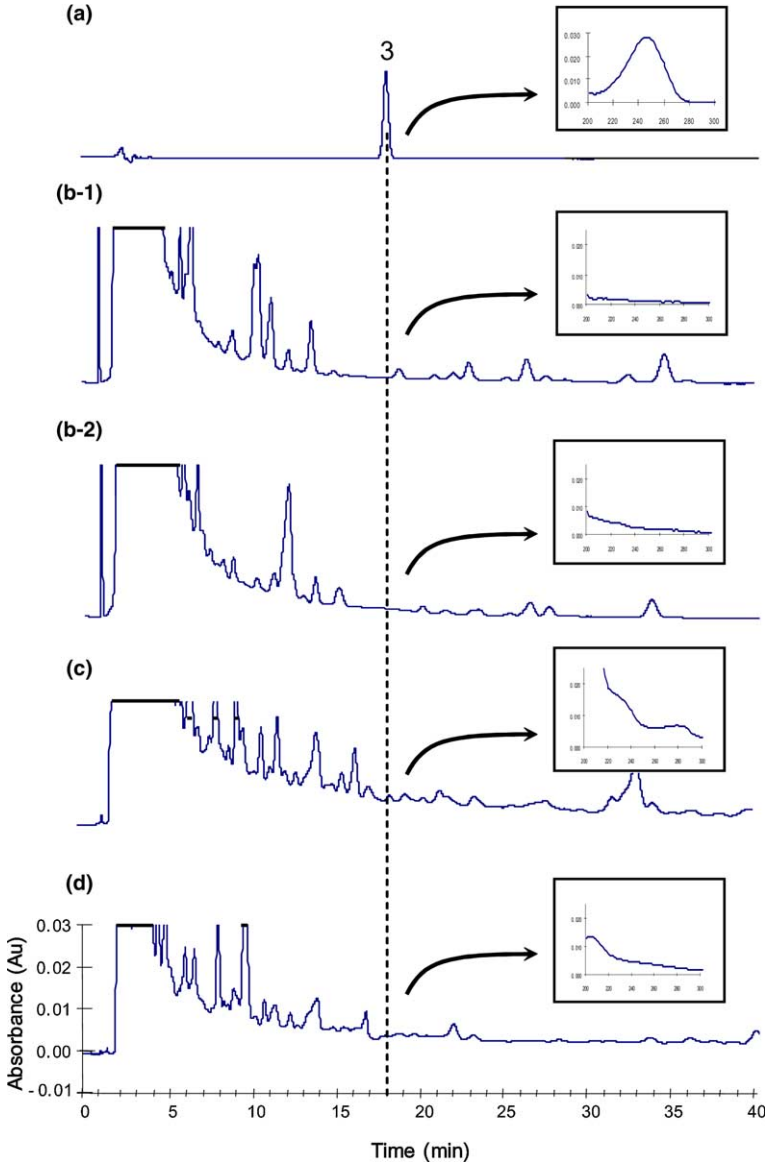

Fig. 4. Typical HPLC chromatograms and PDA spectrum obtained from testosterone standard and pomegranate samples. Concentration levels of standard and sample solutions were as follows: (a) standards (3, testosterone) 0.005 mg/ml; (b-1) pomegranate seed (cultivated in Iran) 1.0 g/ml; (b-2) pomegranate seed (cultivated in Korea) 1.0 g/ml; (c) pomegranate fruit juice 10.0 g/ml; (d) pomegranate preparation 10 g/ml. The peak of interest was shown by both dotted line and its PDA spectrum absorbing range from 200 nm to 300 nm.

separated without overlapping peaks. A reversed phase Capcellpak C_{18} column was chosen for the simultaneous separation of steroid hormones. The detection wavelengths were set at maximum absorption wavelength of each steroid hormones in order to obtain higher sensitivity, and the maximum absorption was 225 nm for estrone and estradiol and 254 nm for testosterone, respectively. Under these conditions, the representative chromatograms of steroid hormones in standard solutions and pomegranate sample solutions were shown in Figs. 2–4, respectively. As shown in Fig. 2, although the peak demonstrated the similar retention time (26.5 min) to estrone standard in pomegranate seed sample (b-1), PDA spectrum of the peak was quite different from that of estrone standard (a). There were no peaks having similar retention time to estrone standard in pomegranate fruit juice (c) and preparation samples (d). As the same manners, there were no detection of estradiol and testosterone from pomegranate samples, respectively (Figs. 3 and 4).

3.2. GC/FID analysis

When a sample might contain a steroid hormones, GC/FID might be applicated for the purpose of the confirmation. As shown in Fig. 5, the steroid estrogens, estrone, estradiol and testosterone were not identified from the three pomegranate samples, respectively (Fig. 5). The GC/FID detection limit (signal to noise ratio of 3:1) of estrone, estradiol and testosterone was 0.005 mg/ml, respectively. The steroid hormones were spiked into the sample before extraction and the recovery range was 102–107%. The peak retention times were as follows: estrone, 13.0 min; estradiol, 13.2 min, testosterone, 10.5 min, respectively. The instrumental precision of peak retention time was obtained by analyzing the peak retention time variation of at least five injections of 0.005 mg/ml of each steroid hormone standards. Due to the numerous components derived from herbal medicines present in concentrated preparations, co-elution of the components might be a provocative problems in HPLC–PDA analysis. There were fortunately no interfering peaks to that of each steroid hormones in GC/FID method. Although the detection limits including peak response were not good, the overall run time for the GC/FID methods was extremely short (15–20 min) in comparison with HPLC methods. This GC/FID method, therefore, might be useful and economical for screening steroid hormones from adulterated natural preparations.

3.3. GC/MS analysis for estrone identification

The use of a highly specific method such as GC/MS was important to provide a confirmatory identification of compounds in the presence of potential interference

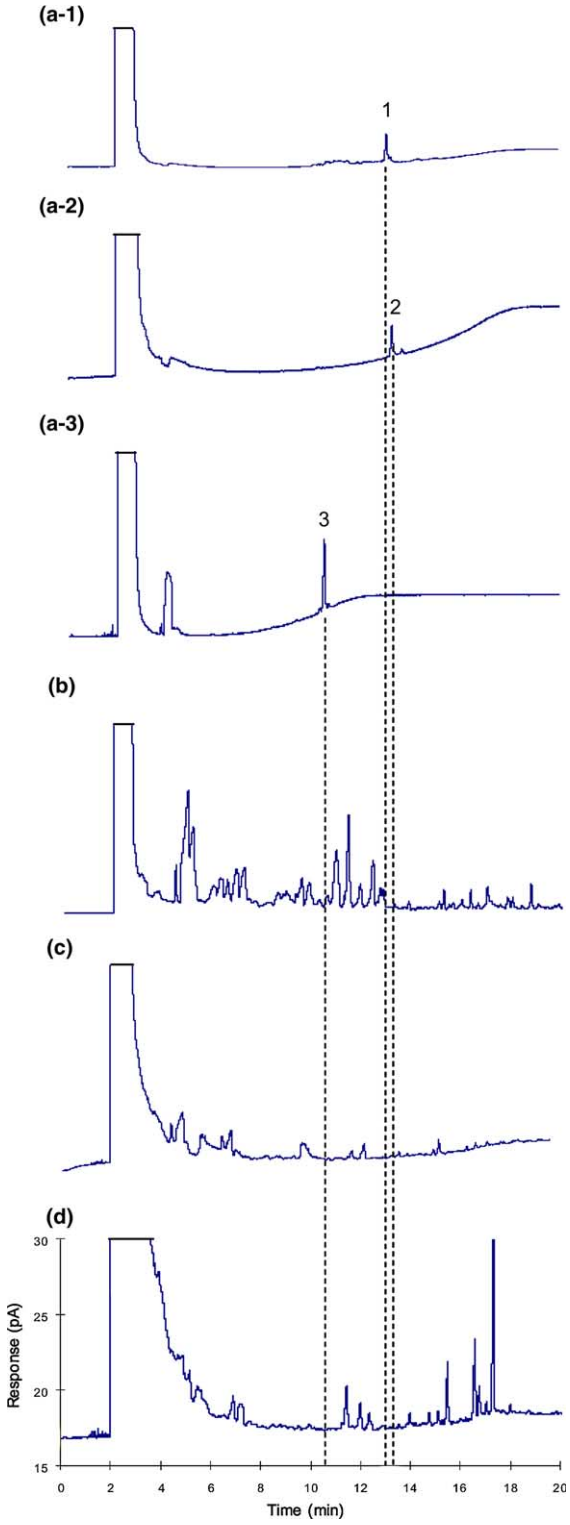

Fig. 5. The GC/FID chromatographic profiles of a steroid hormones and pomegranate samples analyzed according to the method described. Corresponding to a concentration of each steroids were 0.005 mg/ml, respectively: (a) standards (1, estrone; 2, estradiol; 3, testosterone); (b) pomegranate seed 1.0 g/ml; (c) pomegranate fruit juice 10.0 g/ml; (d) pomegranate preparation 10 g/ml. The retention time of compounds of interest was shown by a dotted line.

in the complex matrix. Over the last decades, the combination of mass selective detection with gas chromatography was a commonly used analytical method available for the identification and quantitation of steroids including estrogens (Hartmann & Steinhart, 1997). Due to the numerous components present in pomegranate seeds and fruit juice, co-elution of the components might be a problem. The GC/MS was the most sensitive method of chemical molecular analysis, with detection limits in the nanogram range. The GC/MS total ion chromatograms of estrone standard and the pomegranate samples were presented. As shown in Fig. 6, the eluting peak correspond to estrone was not identified in pomegranate seed, fruit juice and preparation samples by HP 5972 mass selective detector (MSD) coupled with a HP 6890 GC. There, however, was a constituent having similar retention time (12.2 min) exception of different mass spectra of TIC (M^+ 281) to estrone standard (12.2 min, M^+ 270) in the pomegranate seed sample. To identify it more precisely, the most sensitive TMS-derivatized GC/MS analysis was performed. The results showed that the conspicuous constituent detected in pomegranate seed sample was a distinguishable constituent from estrone. Known concentration of estrone standard spiked into the pomegranate seed sample gave a distinctive (splitted) chromatogram with the suspicious constituent. As shown in Fig. 7, although the trace constituent having retention time of 8.8 min was detected in sample (B), the peaks was surely separated by spiking analysis with different two peaks having retention time

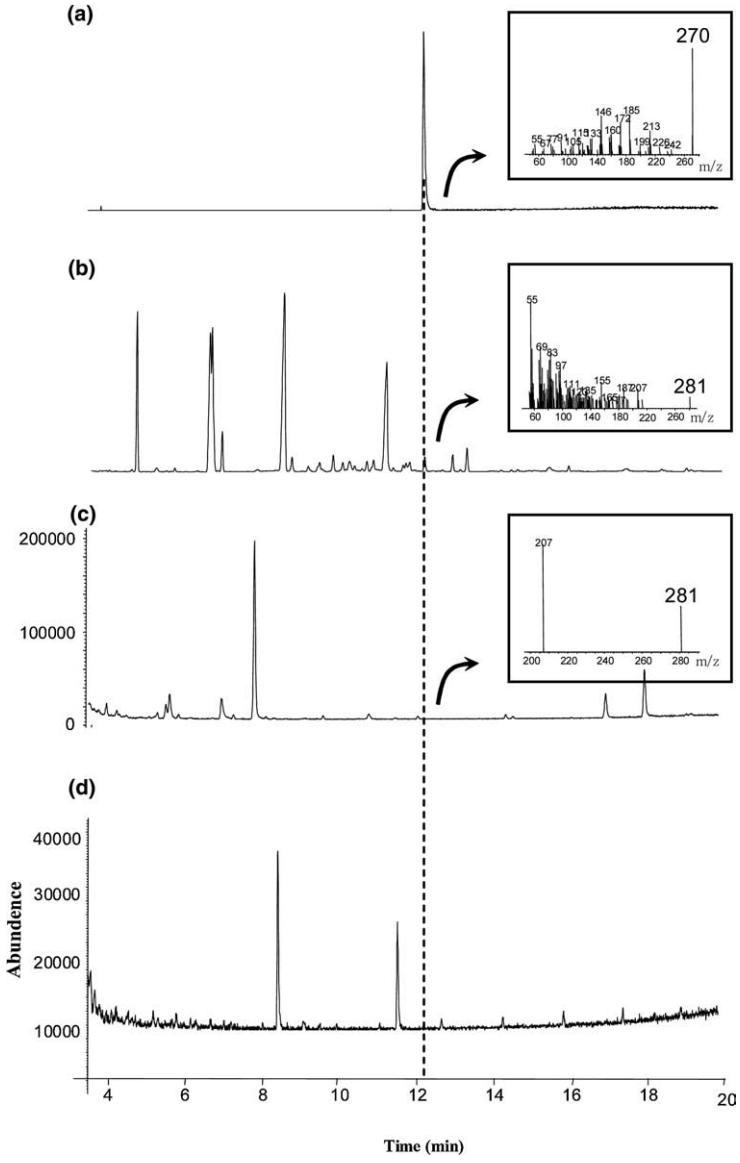

Fig. 6. Detection of estrone in pomegranate samples by HP 5972 GC/MSD showing a total ion chromatogram (TIC) and mass spectrum: (a) standard (1, estrone) 0.005 mg/ml; (b) pomegranate seed (cultivated in Iran) 1.0 g/ml; (c) pomegranate fruit juice 10.0 g/ml; (d) pomegranate preparation 10 g/ml. The peaks of interest were shown by both dotted line and its library searched mass spectrum.

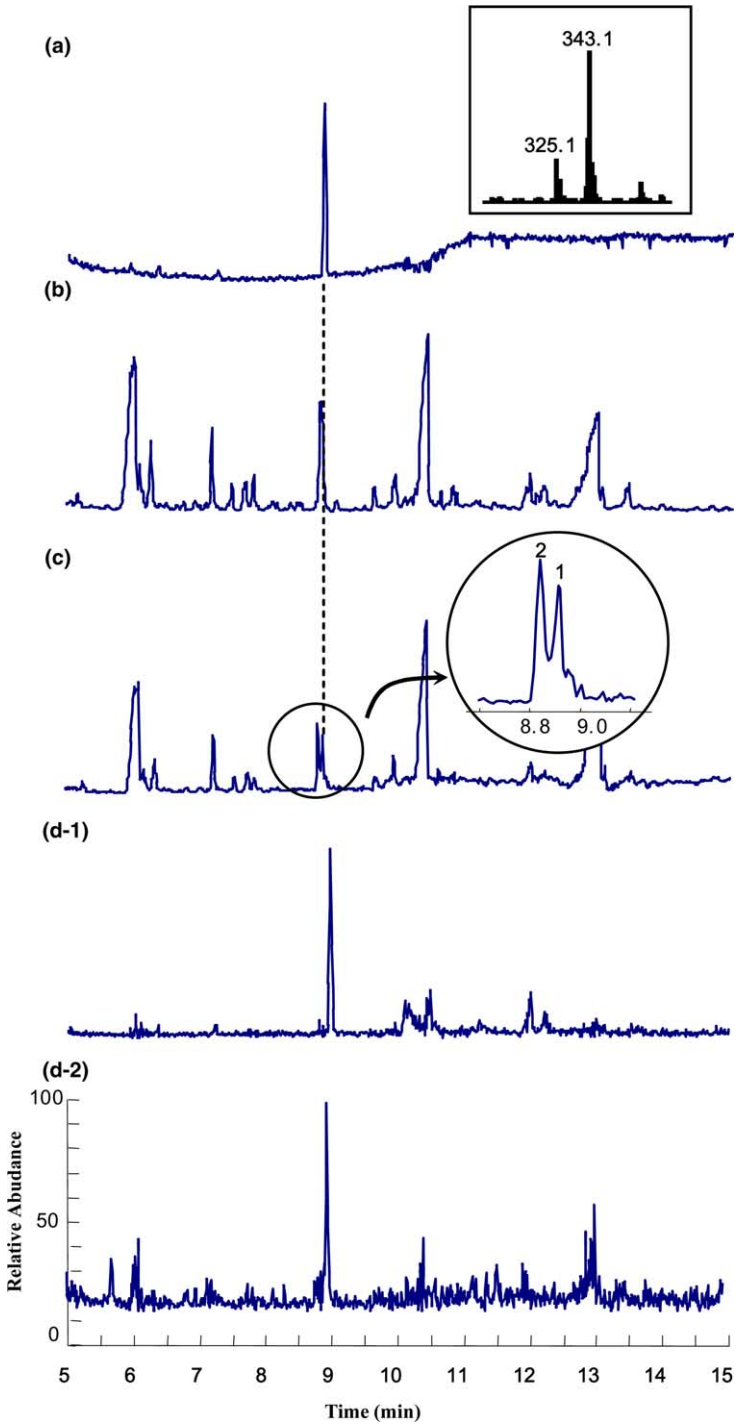

Fig. 7. Detection of estrone in pomegranate seed samples by Finigan Polaris Q GC/MS showing a total ion chromatogram (TIC) and SIM mass: (a) standard (1, estrone) 0.005 mg/ml; (b) pomegranate seed sample 1.0 g/ml; (c) estrone-spiked sample; (d-1) selected ion 343 *m*/*z*; (d-2) selected ion 325. The retention times were as follows: 1, estrone standard 8.9 min; 2, unknown constituent 8.8 min. The constituent of interest was shown by dotted line and splitted chromatogram to unknown constituent and estrone was magnified in circle.

of 8.8 min (unknown) and 8.9 min (estrone), respectively (C). And also, the quantitative results for the comparison of the detected levels was similar to those obtained from HP 5972 mass selective detection. Product ion scans produced a fragmentation dominated by ions at *m*/*z* 325 and 343. No data for selected ion comparison by SIM at *m*/*z* 343 (D-1) and 325 (D-2) were available in pomegranate seed sample, respectively (Fig. 7). Consequently, estrone was not present in pomegranate seed, fruit juice and preparation samples and these results were good agreement with the HPLC–PDA and GC/FID results.

4. Conclusion

This paper described the identification and re-evaluation of several steroid hormones analysis in pomegranate and its commercial preparations using HPLC–PDA, GC/FID and GC/MS. These data suggested that there were no estrone, estradiol and testosterone in pomegranate seed and fruit juice as well as commercial pomegranate preparations. We demonstrated that HPLC with PDA detector was the reliable, rapid and selective method of choice for the accurate analysis of estrone, estradiol and testosterone in pomegranate samples. And also, the GC/MS data showed here were the most conclusive and precise proof compared to what kind of experimental results on identification of steroid hormones in pomegranate until now. Based on illegal adulteration, we examined several commercial pomegranate preparations, simultaneously. As a result, we could not identify the adulterated pomegranate preparations with added synthetic steroid hormones including estrone. However, we have still fears for adulteration upon pomegranate preparations circulated in marketplace because woman seek for them. We suggested that these HPLC and GC/MS methods might be a valuable and decisive tool for both pomegranate researchers and the managing authorities on illegal food and medicines.

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