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Article *in* Journal of Pharmaceutical and Biomedical Analysis · February 2019

DOI: 10.1016/j.jpba.2018.12.045

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The power of hyphenated chromatography—Time of flight mass spectrometry for unequivocal identification of spirostanes in bodybuilding dietary supplements

Bharathi Avula[a,1], Amar G. Chittiboyina[a,1], Ji-Yeong Bae[a], Saqlain Haider[a], Yan-Hong Wang[a], Mei Wang[a], Jianping Zhao[a], Patricia A. Deuster[b], Ikhlas A. Khan[a,c,*]

[a] National Center for Natural Products Research, School of Pharmacy, The University of Mississippi, University, MS, 38677, USA
[b] Consortium for Health and Military Performance, Department of Military and Emergency Medicine, Uniformed Services University, 4301 Jones Bridge Road, Bethesda, MD, 20814, USA
[c] Division of Pharmacognosy, Department of BioMolecular Sciences, School of Pharmacy, The University of Mississippi, MS 38677, USA

ARTICLE INFO

Article history:
Received 4 October 2018
Received in revised form
11 December 2018
Accepted 29 December 2018
Available online 1 February 2019

Keywords:
5α-Hydroxy laxogenin
UHPLC-QToF-MS
Diosgenin
Spirostanes
Anabolic steroids

ABSTRACT

A previously unidentified purported botanical ingredient was found in dietary supplements marketed for anabolic benefits. In an attempt to assess the 'naturalness' of a group of steroid-like compounds called laxogenins, a UHPLC-QToF method was developed. Several dietary supplements claim to contain 5α-hydroxy laxogenin, which is a derivative of a naturally occurring spirostane-type steroid, laxogenin. Although laxogenin has been isolated from the rhizomes of *Smilax sieboldii*, 5α-hydroxy laxogenin has not been isolated or reported from any natural source. These derivatives of laxogenins have untested anabolic properties. Due to the low UV absorbance of the spirostanes, a mass spectrometric method in positive ion mode was developed for unambiguous identification of laxogenin and closely related compounds. To show the utility of the developed method, twelve dietary supplements labeled to contain 5α-hydroxy laxogenin or laxogenin as 5α-hydroxy laxogenin were analyzed as a proof-of-concept. Five supplements did not contain any 5α-hydroxy laxogenin, whereas in the remaining seven samples, spirostane-type contaminants were identified along with the labeled 5α-hydroxy laxogenin. The identity of some of these contaminants was established based on reference standards along with mass fragmentation patterns. One of the unlabeled contaminants was identified as the phytosteroid saponin, diosgenin, a common starting precursor of several steroidal drugs. Several synthetic derivatives of diosgenin were identified in the eight products. These findings indicate that the labeled 5α-hydroxy laxogenin along with other spirostanes found in supplements are synthetic and signify a lack of quality controls. Additionally, an unlabeled, anabolic androgenic steroid, arimistane, an aromatase inhibitor, was also identified in one product. Laxogenin, was not detected in any of the samples analyzed during this investigation.

1. Introduction

The United States Congress has authorized the FDA by virtue of the Dietary Supplement Health and Education Act (DSHEA) along with subsequent amendments to require companies to submit a new dietary ingredient notification (NDIN) if they planned to market a dietary supplement product that contains a ñew dietary ingredient (NDI).Än NDI is defined as any ingredient that was not on the market prior to 1994 and meets the requirements of Section 413(d). The notification must provide information showing the new dietary ingredient will "reasonably be expected to be safe under the conditions of use recommended or suggested in the labeling." After passage of DSHEA and despite the NDIN requirements a few unscrupulous companies continue to introduce new dietary ingredients and actively market them for various purposes.

In early 2014, a product containing two previously unfamiliar ingredients were identified, and were considered for further analysis. The only two ingredients listed on the Supplement Facts panel of the product were Carbopol® and 5α-hydroxy laxogenin (Fig. 1, structure **2**). Carbopol® is a water soluble polymer, used to emulsify and/or stabilize products or in drugs and dietary supplements for controlled release of the primary ingredient. Review of various

* Corresponding author at: National Center for Natural Products Research, The University of Mississippi, University, MS, 38677, USA.
E-mail address: ikhan@olemiss.edu (I.A. Khan).
[1] Authors share equal contribution.

https://doi.org/10.1016/j.jpba.2018.12.045
0731-7085/

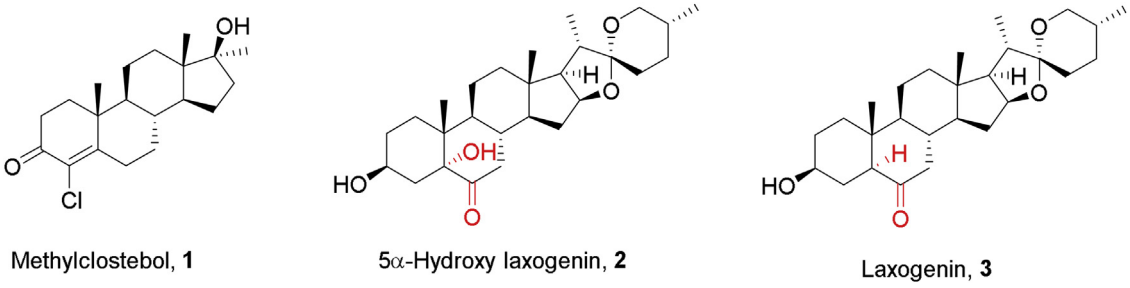

Fig. 1. Structures of the anabolic steroid and laxogenins.

sources indicated 5α-hydroxy laxogenin was being promoted as an alternative to anabolic steroids. Finally, a search of the FDA NDI database indicated that the 5α-hydroxy laxogenin had not been through the NDIN process.

Our preliminary analysis of one dietary supplement product resulted in identification of several interesting, unusual ingredients. Thorough analysis of this product showed that many ingredients that have been banned by World Anti-Doping Agency (WADA) were identified including ingredients considered illegal or controlled substances as well as anabolic androgenic steroids (AAS). These ingredients were of concern because the concentrations of some AAS, in particular methylclostebol (Fig. 1, structure **1**) [1], were well above what might be considered a "contaminant" level.

Methylclostebol [1] is a synthetic, orally active designer AAS first used as a performance enhancing drug in the 1960s; it is now on the WADA's banned list. The analyses indicated the amount of methylclostebol was 20 mg/g or almost 30 mg per serving. The large quantities of AAS, the undefined biological function and origin of 5α-hydroxy laxogenin and the ready availability of these products online, prompted us to investigate the source of 5α-hydroxy laxogenin or laxosterone® listed on the label. Structurally, 5α-hydroxy laxogenin (Fig. 1, structure **2**) is very similar to laxogenin (Fig. 1, structure **3**). Laxogenin was first identified in 1965 by a Japanese laboratory as a substance in the plant - *Smilax sieboldii* [2], which is native to Japan, Korea, and China. Laxogenin *per se* is one of many plant-based spirostane-type steroids, known as brassinosteroids and identified as one of the phytochemicals derived from *Allium schoenoprasum* [3], *Allium chinense* [4], *Solanum unguiculatum* [5] or *S. sieboldii* in minor quantities [2]. On the other hand, there is no report on the natural existence of 5α-hydroxy laxogenin and it is indeed reported to be a synthetic derivative of diosgenin [2]. In fact, diosgenin is used as an industrial starting raw material for more than 50% of the commercial synthetic steroidal drugs, e.g. progesterone, cortisone, pregnenolone, testosterone and other steroids [2,3]. Diosgenin has been extensively modified to yield chemically diverse analogs with a wide variety of biological activities including plant growth promotion [4]. Laxogenin has been touted to promote growth and vitality in plants and possesses a similar anabolic/androgenic ratio to the popular oral anabolic steroid, anavar (or oxandrolone). Moreover, laxogenin has been actively marketed to possess no liver toxicity or test positive for steroids, and is marketed as the same compound as 5α-hydroxy laxogenin. The confusion arises from an obscure Russian research study [5] wherein 6-keto derivatives of the natural saponins - agigenin, diosgenin and alliogenin - were investigated for their anabolic, but not androgenic activities. These issues, coupled with lack of information on either laxogenin or 5α-hydroxy laxogenin in the Food and Drug Administration's NDIN database created a strong interest in probing the quality of purported laxogenins and the label claims used in marketing them as dietary supplements.

2. Materials and methods

2.1. Reference standards and chemicals

Laxogenin was acquired from Proactive Molecular Research (Gainesville, FL, USA). Piperine was purchased from Sigma (St. Louis, MO, USA). Diosgenin was procured from MP Biomedicals Inc. (Ohio, USA). Arimistane was procured from Steraloids, Inc. (Newport, RI, USA). 5α-Hydroxy laxogenin was procured from ARK Pharm. Inc. (Arlington Heights, IL, USA). 5-Methyl-7-methoxyisoflavone and PABA (*para*-aminobenzoic acid) were procured from Extrasynthese (Genay Cedex, France) and Alfa Aesar (Haverhill, MA), respectively. Acetonitrile and formic acid were HPLC grade purchased from Fisher Scientific (Fair Lawn, NJ, USA). Water for the LC mobile phase was purified using a Milli-Q system (Millipore). All dietary supplements (DS) were commercially available and were purchased online. The active ingredients declared by the manufacturers are listed in Table 2.

2.2. Synthesis of spirostane-type reference standards

25(*R*)-5α,6α-Epoxyspirostan-3β-ol (Fig. 2, structure **5a**), (3β,5β,6β,25R)-5,6-epoxyspirostan-3-ol (Fig. 2, structure **5b**), anzurogenin D (Fig. 2, structure **6**), Δ4-diosgenone (Fig. 2, structure **7**), (5α,25R)-5-hydroxyspirostan-3,6-dione (Fig. 2, structure **8**) and diosgenin-3,6-dione (Fig. 2, structure **9**) were synthesized at the Natural Center for Natural Products Research (The University of Mississippi, University, MS, USA) and the details are listed below. The identity of these compounds were confirmed by NMR, ESI-HRMS data and the purity of these compounds was established with UHPLC and found to be > 95%.

2.3. Preparation of 5,6-epoxyspirostan-3-ols (Fig. 2, structures ***5a*** *and* ***5b***)

Epoxidation was done according to the previously reported procedure [6]. A solution of diosgenin (Fig. 2, structure **4**) (100 mg, 0.24 mmol) in anhydrous DCM (20 ml) at 0 °C was treated with *m*-chloroperbenzoic acid (88 mg, 0.51 mmol). The reaction mixture was stirred for 24 h at room temperature. After completion, the reaction was washed successively with aqueous Na_2SO_3 (10%, 5 mL), $Na_2S_2O_3$ (5%, 5 mL), saturated $NaHCO_3$ (10 mL) and brine (10 mL). The DCM layer was dried over anhydrous Na_2SO_4 and concentrated. The crude mixture was purified over silica gel by column chromatography by using EtOAc: Hexanes (40:60) to yield a diastereomeric mixture of α- and β-epoxides (Fig. 2, structures **5a** and **5b**) and the analytical data (^{1}H NMR and MS) were identical to the data reported in literature [6].

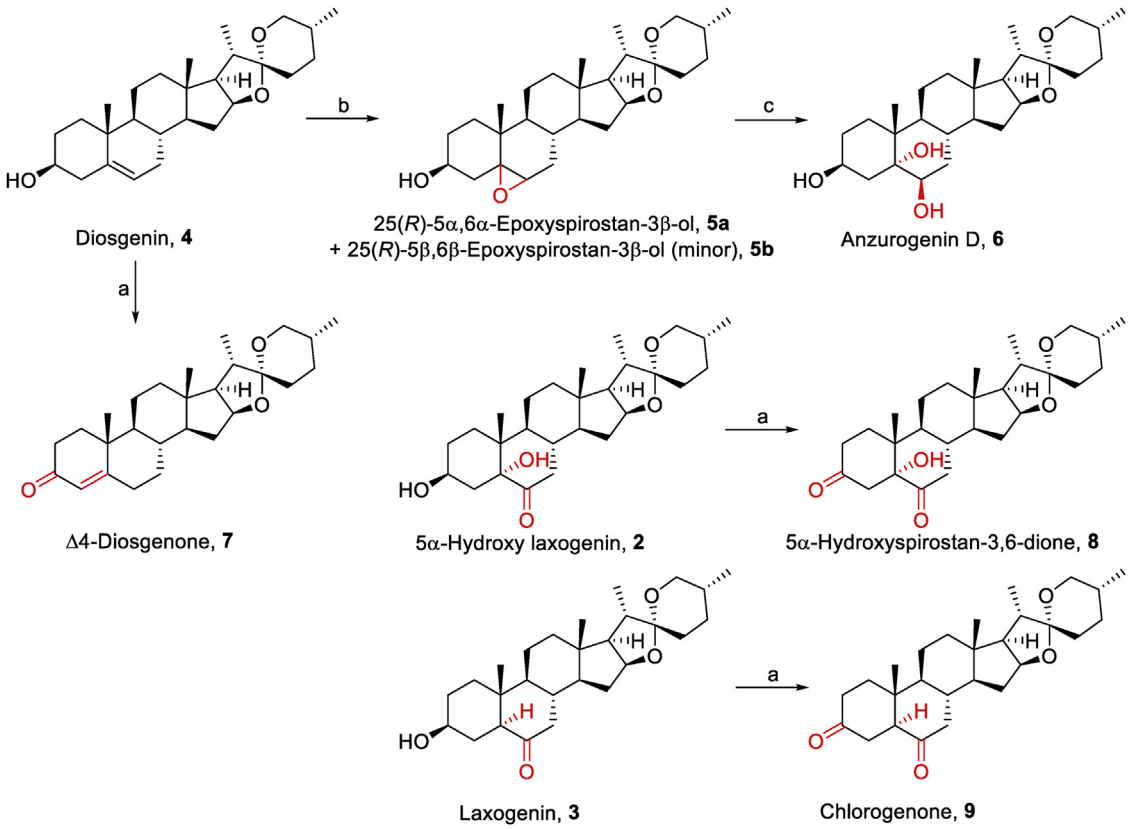

Fig. 2. Schematic representation of 5α-hydroxy laxogenin and related compounds found in nine dietary supplements along with their syntheses. *Reagents and Conditions:*a). Dess-Martin periodinane, CH_2Cl_2, 0°C to r.t., 2h; b). *m*-Chloroperbenzoic acid, $NaHCO_3$, DCM, 0°C to r.t., 24h; c). Dowex® 50WX2, MeOH:H_2O (1:1 v/v), reflux, 2h.

2.4. *Preparation of Anzurogenin* D *(Fig. 2, structure **6**)*

To a solution of 5,6-epoxyspirostan-3-ols (Fig. 2, structures **5a** and **5b**) (72 mg, 0.72 mmol) in aqueous MeOH (16 ml, 1:1 v/v), DOWEX 50 W X2 (72 mg) was added and heated to reflux. After 4 h, the reaction mixture was cooled to room temperature, filtered, concentrated and extracted with chloroform (2 × 10 mL). The combined organic layers were dried over anhydrous Na_2SO_4, concentrated and the resulting crude mixture was purified by column chromatography over silica gel using EtOAc:Hexanes (70:30) to yield anzurogenin D (Fig. 2, structure **6**) as a **white** solid. The spectral data was in agreement with reported literature data [6,7].

2.5. *Preparation of Δ^4-diosgenone (Fig. 2, structure **7**)*

To a solution of diosgenin (Fig. 2, structure **4**) (60 mg, 1.4 mmol) in anhydrous DCM (20 mL) at 0° C, Dess–Martin periodinane (65 mg, 1.5 mmol) was added and the mixture was stirred at room temperature for 2 h. The reaction mixture was filtered through celite and purified by column chromatography over silica gel using EtOAc: Hexanes (10:90) to yield Δ^4-diosgenone as a **white** solid with analytical data identical to the reported data [7].

2.6. *Preparation of 5α -hydroxyspirostan-3,6-dione (Fig. 2, structure **8**)*

To a solution of 5α-hydroxy laxogenin (Fig. 2, structure **2**) (20 mg, 0.45 mmol) in anhydrous DCM (15 mL) at 0° C, Dess–Martin periodinane (19 mg, 0.45 mmol) was added and the resulting mixture was stirred at room temperature for 2 h, evaporated and purified over silica gel chromatography to furnish 5α-hydroxyspirostan-3,6-dione as a pure **white** solid. The ^{1}H NMR and MS data was in agreement with reported data [8].

2.7. *Preparation of diosgenin-3,6-dione (Fig. 2, structure **9**)*

To a solution of laxogenin (Fig. 2, structure **3**) (44 mg, 1 mmol) in anhydrous DCM (15 mL) at 0 °C, Dess–Martin periodinane (40 mg, 1 mmol) was added and the resulting mixture was stirred at room temperature for 2 h, evaporated and the resulting residue was purified over silica gel to furnish diosgenin-3,6-dione as a pure **white** solid. The ^{1}H NMR and MS data were in agreement with reported data [9].

2.8. *Sample preparation*

Standard compounds of stock solutions were prepared at a concentration of 1 mg/mL. All standards were prepared in a concentration of 10 μg/mL. An adequate amount of capsule contents (average weight of dosage form, Table 2) was sonicated in methanol (2.5 mL) for 30 min followed by centrifugation for 15 min at 959 × g. The supernatant was transferred to a 10 mL volumetric flask. The procedure was repeated three more times and the respective supernatants were combined. The final volume was adjusted to 10 mL with methanol and mixed thoroughly. Prior to injection, an adequate volume (ca. 2 mL) was passed through a 0.45 μm PTFE membrane filter. The first 1.0 mL was discarded and the remaining volume was collected in an LC sample vial.

Table 1
Retention time, MS, MS/MS, molecular formula and compound name of diosgenin and other spirostanes using UHPLC-QToF-MS in positive ESI mode (calculated mass is represented in parentheses).

#	Rt (min)	*m/z* $[M+H]^+$	*m/z* $[M+Na]^+$	*m/z* $[2M+Na]^+$	Key Fragment Ions (35 eV)	Molecular Formula	Compound Identity
1	3.0	463.3055 (463.3054)	485.2851 (485.2874)	947.5870 (947.5855)	393.2655; 335.2183; 191.1057	$C_{27}H_{42}O_6$	Unknown-1
2	3.1	449.3260 (449.3262)	471.3063 (471.3081)	919.6273 (919.627)	305.2086; 287.1998; 269.1889; 251.1785; 157.1003	$C_{27}H_{44}O_5$	Anzurogenin D (6)
3	4.3	447.3104 (447.3105)	469.2912 (469.2924)	915.5962 (915.5957)	303.1924; 285.1828; 267.1734; 257.1899; 249.1622; 239.1785	$C_{27}H_{42}O_5$	5α-Hydroxy laxogenin (2)
4	4.7	431.3156 (431.3156)	453.2957 (453.2975)	883.6071 (883.6058)	253.1951; 211.1477; 157.1010	$C_{27}H_{42}O_4$	Unknown-2
5	6.0	431.3154 (431.3156)	453.2947 (453.2975)	883.6077 (883.6058)	253.1949; 211.147; 157.0995	$C_{27}H_{42}O_4$	Unknown-3
6	6.2	431.3144 (431.3156)	453.2963 (453.2975)	883.6071 (883.6058)	413.3012; 287.1974; 269.1881; 251.1775	$C_{27}H_{42}O_4$	Laxogenin (3)
7	6.3	429.2999 (429.2999)	451.2813 (451.2819)	879.5745 (879.5745)	285.1849; 267.1738; 133.1004	$C_{27}H_{40}O_4$	Unknown diosgenin analog-1
8	6.4	445.2947 (445.2949)	467.2766 (467.2768)	911.564 (911.5644)	427.2812; 409.2700; 301.1785; 283.1679; 265.1576; 255.1730; 237.1629	$C_{27}H_{40}O_5$	(5α, 25*R*)-5-Hydroxyspirostan-3,6-dione (8)
9	6.5	429.2995 (429.2999)	451.2812 (451.2819)	879.5741 (879.5745)	285.1835; 267.1734; 133.0997	$C_{27}H_{40}O_4$	Chlorogenone (9)
10	7.8	431.3149 (431.3156)	453.2960 (453.2975)	883.6061 (883.6058)	287.2016; 269.1897; 251.1788; 184.0725	$C_{27}H_{42}O_4$	25(*R*)-5β,6β-Epoxyspirostan-3β-ol (5b)
11	8.1	431.3150 (431.3156)	453.2965 (453.2975)	883.6063 (883.6058)	287.2002; 269.1900; 251.1783; 184.0726	$C_{27}H_{42}O_4$	25(*R*)-5α,6a-Epoxyspirostan-3β-ol (5a)
12	9.1	427.2842 (427.2843)	449.2643 (449.2662)	875.543 (875.5432)	409.2685; 283.1653; 184.0725	$C_{27}H_{38}O_4$	Diosgenin-3,6-dione (10)
13	11.5	415.3204 (415.3207)	437.3013 (437.3026)	851.6131 (851.616)	271.2058; 253.1953; 238.1713; 211.1480; 157.1017	$C_{27}H_{42}O_3$	Diosgenin (4)
14	12.4	413.3042 (413.305)	435.2859 (435.287)	847.584 (847.5847)	269.1739; 184.0585	$C_{27}H_{40}O_3$	Unknown diosgenin analog-2
15	12.6	413.3037 (413.305)	435.2854 (435.287)	847.5842 (847.5847)	269.1753; 184.0594	$C_{27}H_{40}O_3$	Δ^4-Diosgenone (7)

2.9. Instrumentation, analytical conditions and data processing

Ultra-high performance liquid chromatography-quadrupole time of flight-mass spectrometry (UHPLC/QToF-MS)

The liquid chromatographic system was an Agilent Series 1290 comprised of the following modular components: binary pump, a vacuum solvent micro degasser, an autosampler with 100-well tray and a temperature controlled column compartment. Separation was achieved on an Agilent SB-C8 (2.1 × 100 mm, 1.8 μm) column. The mobile phase consisted of water (0.1% formic acid) (A) and acetonitrile with 0.1% formic acid (B) at a flow rate of 0.23 mL/min. The separation was achieved with the following gradient: 0 min, 45% A: 55% B to 60% B in next 5 min then for next 6 min 90% B, then to 100% B in next 4 min. Separation was followed by a 5 min washing procedure with 100% B and a re-equilibration period of 5 min. Two microliters of sample were injected. The column temperature was 40 °C.

The mass spectrometric analysis was performed with a QToF-MS/MS (Model #G6530 A, Agilent Technologies, Santa Clara, CA, USA) equipped with an ESI source with Jet Stream technology connected with a nitrogen generator (Peak) using the following parameters: drying gas (N_2) flow rate, 11 L/min; drying gas temperature, 300 °C; nebulizer, 30 psig, sheath gas temperature, 300 °C; sheath gas flow, 9 L/min; capillary, 3500 V; skimmer, 65 V; Oct RF V,

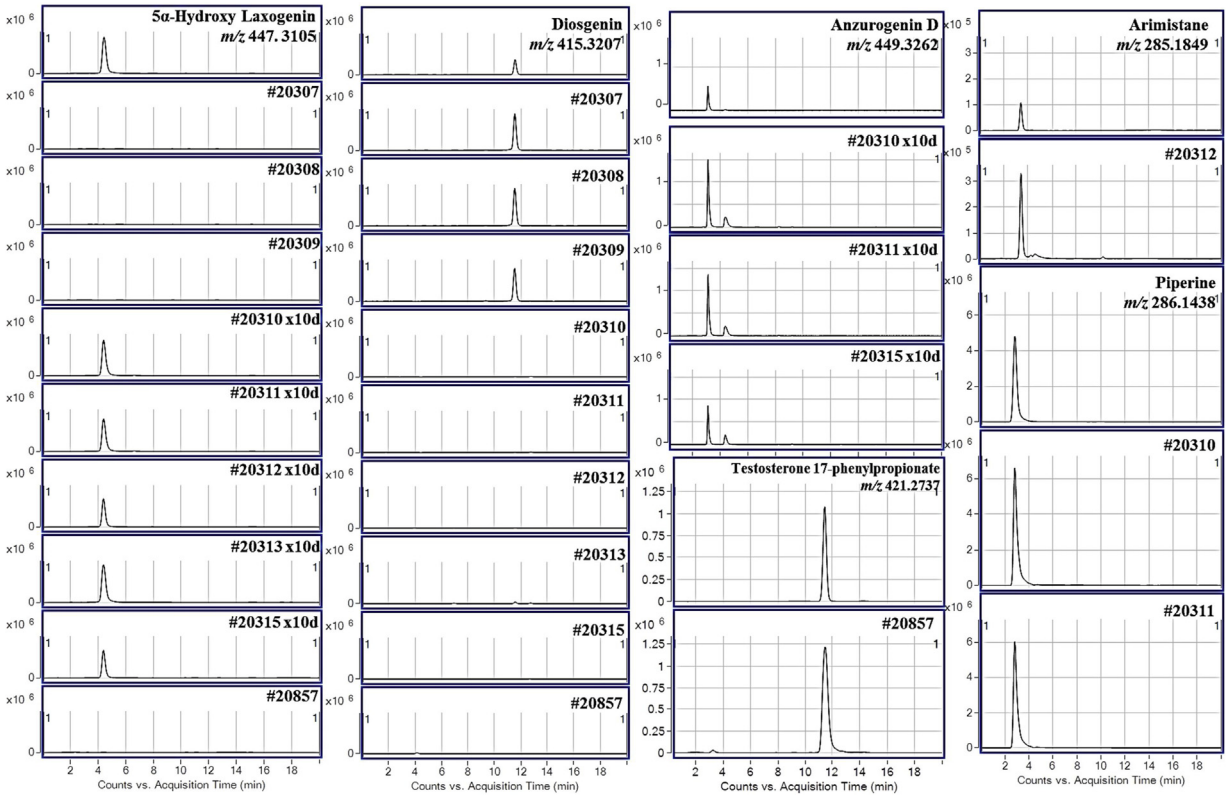

Fig. 3. Extracted ion chromatograms of standard compounds and dietary supplements using LC-QToF-MS.

750 V; fragmentor voltage, 125 V. The sample collision energy was set at 35 eV. Total analysis time was 15 min. ESI-QTOF-MS analysis was performed in 2 GHz extended dynamic range, positive ionization mode. The identification of compounds present in samples was performed by comparison with reference standards. The tentative identification of some derivatives was based on the accurate mass spectrum.

All the operations, acquisition and analysis of data were controlled by Agilent MassHunter Acquisition Software Ver. A.05.00. Each sample was analyzed in positive mode in the range of m/z = 50-1500. Accurate mass measurements were obtained by means of ion correction techniques using reference masses at m/z 121.0509 (protonated purine) and 922.0098 [protonated hexakis (1H, 1H, 3H-tetrafluoropropoxy) phosphazine or HP-921] in positive ion mode. The compounds were confirmed in each spectrum. For this purpose, the reference solution was introduced into the ESI source via a T-junction using an Agilent Series 1200 isocratic pump (Agilent Technologies, Santa Clara, CA, USA) using a 100:1 splitter set at a flow rate of 20 μL/min. The LOD (limits of detection) were found to be between 50–500 ng/mL for all reference compounds analyzed.

3. Results and discussion

When a manufacturer or distributor of dietary supplements plans to market a product containing a ñew dietary ingredientẗhey must notify the Food and Drug Administration and provide information that the "new dietary ingredient will reasonably be expected to be safe under the conditions of use recommended or suggested in the labeling" [10]. This FDA regulation, the natural existence of laxogenin, the misrepresentation with 5α-hydroxy laxogenin, and lack of NDIN submission information in the FDA database for laxogenin or 5α-hydroxy laxogenin prompted us to probe into the quality of laxogenin ingredients readily available in dietary supplements. These dietary supplements are specifically marketed to people interested in bodybuilding for improving muscle protein synthesis and overall muscle mass. These biologic and functional claims of being the "premier natural muscle building supplements on the market" are based solely on data showing that steroid saponins derived from laxogenin exhibited plant growth-promoting activity in the radishes through elongation of hypocotyls and greater weight of cotyledons (*Raphanus sativus*) [4]. Paradoxically, the plant growth-promoting data were insignificant, independent of tested concentrations and identical to control experiments, yet two laxogenin analogs were identified as plant growth promoters. To assess the quality of the ingredients in supplements, twelve products with the ingredient "laxogenin" or "laxosterone" on their label were selected for analysis. In this paper, we report the analyses of the products and discuss possible problems with those products purporting to contain laxogenin on the product's label.

In order to achieve sufficient resolution of the analytes and identification of compounds by using accurate mass, an UHPLC-QToF in positive ESI method was developed in full scan MS and MS-MS mode. Accurate mass determination leads to chemical formula identification, which can provide structural information when forming product ions with MS-MS. Although MS can often distinguish overlapping peaks through extracted ion chromatograms (EIC), in some incidences, viz., structural, geometrical isomers will have the same m/z values and cannot be distinguished by EIC. In such cases, chromatography will aid in successful identification of both targeted and untargeted analytes. MassHunter Workstation software, including Qualitative Analysis (version B.07.00), was used for processing raw data, including background subtraction, data filtering, molecular feature extraction and molecular formula estimation. To perform subtraction of molecular features (MFs) originating from the background, analysis of a blank sample (methanol) was carried out under identical instrument settings and background MFs were removed. MFs were characterized by

Table 2
Qualitative analysis of laxogenin containing twelve dietary supplements based on label claim.

#	Code #	Ingredients listed on the label by the manufacturer	Weight (mg)	Analysis results	Exogenous components detected[#]
1	20190	5α-Hydroxy Laxogenin	511.3	ND	1) Diosgenin (**4**) 2) Δ^4-Diosgenone (**7**) 3) Glyceryl fatty acid esters 4) Unknown steroidal compounds 5) Methoxyisoflavone(5-Methyl-7-methoxyisoflavone)
2	20307	5α-Hydroxy Laxogenin	505.5	ND	1) Diosgenin (**4**) 2) Δ^4-Diosgenone (**7**) 3) Glyceryl fatty acid esters 4) Unknown steroidal compounds 5) Methoxyisoflavone(5-Methyl-7-methoxyisoflavone)
3	20308	5α-Hydroxy Laxogenin	527.3	ND	1) Diosgenin (**4**) 2) Δ^4-Diosgenone (**7**) 3) Glyceryl fatty acid esters 4) Unknown steroidal compounds
4	20309	Laxogenin as 5α-Hydroxy Laxogenin	499.9	ND	1) Diosgenin (**4**) 2) Δ^4-Diosgenone (**7**) 3) Glyceryl fatty acid esters 4) Unknown steroidal compounds 5) Methoxyisoflavone(5-Methyl-7-methoxyisoflavone)
5	20310	Laxogenin as 5α-Hydroxy Laxogenin	545.8	+ +	1) 25(*R*)-5α,6α-Epoxyspirostan-3β-ol (**5a**) 2) 25(*R*)-5β,6β-Epoxyspirostan-3β-ol (**5b**) 3) Anzurogenin D (**6**) 4) (5α,25*R*)-5-Hydroxyspirostan-3,6-dione (**8**) 5) Unknown diosgenin analogs 6) Piperine
6	20311	5α-Hydroxy Laxogenin Piperine	540	+ +	1) 25(*R*)-5α,6α-Epoxyspirostan-3β-ol (**5a**) 2) 25(*R*)-5β,6β-Epoxyspirostan-3β-ol (**5b**) 3) Anzurogenin D (**6**) 4) (5α,25*R*)-5-Hydroxyspirostan-3,6-dione (**8**) 5) Unknown diosgenin analogs
7	20312	5α-Hydroxy Laxogenin	515.9	+	1) Androsta-3,5-diene-7,17-dione (Arimistane) 2) Piperine
8	20313	5α-Hydroxy Laxogenin	378.9	+	1) Diosgenin (**4**) 2) 25(*R*)-5α,6α-Epoxyspirostan-3β-ol (**5a**) 3) 25(*R*)-5β,6β-Epoxyspirostan-3β-ol (**5b**) 4) Δ^4-Diosgenone (**7**) 5) Disogenin-3,6-dione (**10**)
9	20315	5α-Hydroxy Laxogenin Piperine 5α-Hydroxy Laxogenin Piperine	355.6	+ + + +	1) Anzurogenin D (**6**) 2) Δ^4-Diosgenone (**7**) 3) (5α,25*R*)-5-Hydroxyspirostan-3,6-dione (**8**) 4) Disogenin-3,6-dione (**10**) 5) *para*-Aminobenzoic acid
10	20833	Rosemary extract *N*-acetyl-*L*-cysteine Stinging nettle extract 5α-Hydroxy Laxogenin	795	+ + + +	1) Anzurogenin D (**6**) 2) (5α,25*R*)-5-Hydroxyspirostan-3,6-dione (**8**)
11	20834	Turmeric extract *N*-acetyl-*L*-cysteine Piperine	766.6	ND + +	1) Anzurogenin D (**6**)
12	20857	5α-Hydroxy Laxogenin 6, 7-Dihydroxybergamottin Piperine	552.9	ND + +	1) DHEA (Dehydroepiandrosterone) 2) Testosterone phenylpropionate

ND = Not Detected; + = detected; # These components were not listed on the label. Figure-3 illustrate the possible synthetic source of some of these exogenous components.

retention time, intensity at the apex of the chromatographic peak and accurate mass.. The large amount of raw data possibly representing the compounds was converted to useful information using the molecular feature extraction algorithm (MFE) from the Mass Hunter Qualitative Analysis software version B.07.00 (Agilent) was used. MFE is a chromatographic deconvolution algorithm to find all components in the sample which cleans data background ion noise, resolves co-eluting interferences and extracts the list of the potential compounds. The data were processed using this algorithm to find chromatographic peaks which include adducts such as Na^+, and dimers. The MFE parameter settings were optimized and the following were applied: single charge state of the analyzed ions, compound quality score of >80, more than >5000 counts for compound filter, target data type of common organic molecules with peak spacing tolerance 0.0025 *m/z*. False positives were eliminated and compounds were verified based on the HRMS data. Additionally, the accurate masses of the extracted features (by MFE) were compared to the exact masses of compounds in the

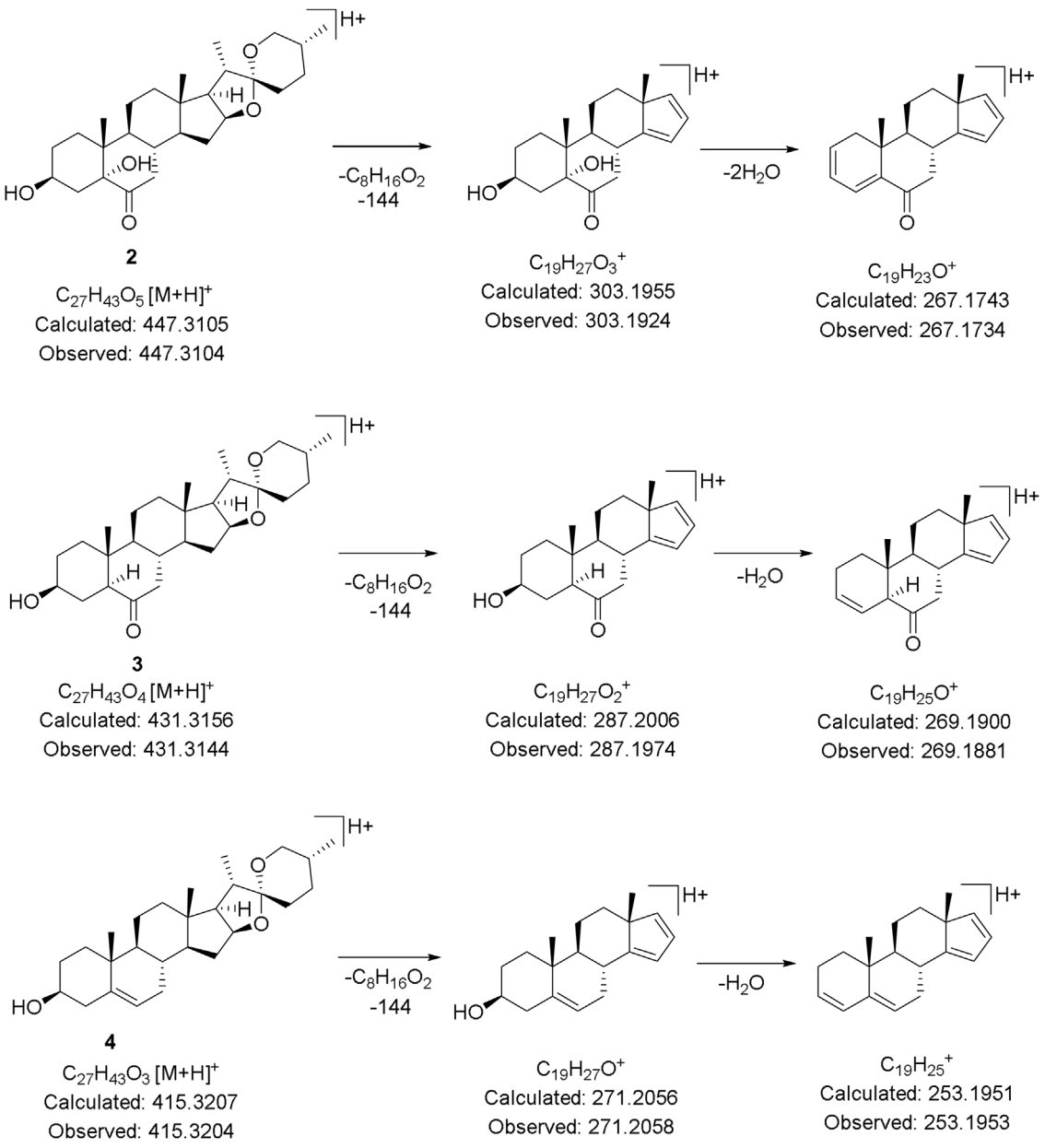

Fig. 4. Proposed fragmentation pathway for 5α-hydroxy laxogenin (**2**), laxogenin (**3**) and diosgenin (**4**).

steroids database (241 compounds), METLIN database and Agilent Tox database (8998 compounds) using mass tolerance of 10 ppm.

The intensity of the peak was selected above the limit of detection for 5α-hydroxy laxogenin, diosgenin and anzurogenin D as 0.05 μg/mL, 0.5 μg/mL, 0.1 μg/mL, respectively.

Considering the complex constituents of the samples and non-polar nature of these compounds, a C8 column was selected to provide sufficient resolution. In addition, formic acid at 0.1% was introduced into the mobile phase to alleviate peak tailing and improve ionization. The total ion chromatograms (TIC) of all compounds were investigated. Table 1 shows the MS and MS-MS data for all compounds detected in positive ESI mode along with the proposed molecular formulae, calculated masses and retention times. Identification of these compounds by using UHPLC-QToF-MS was performed with the reference standards. Some peaks contained the same protonated molecules in the MS spectra and similar product ions in MS/MS spectra, but had different retention behaviors. These studies were performed by extracting $[M+H]^+$ ions. The major fragments were obtained for the compounds by means of collision-induced dissociation (CID) of the protonated molecule and compared with similar data produced for standard compounds under the same conditions (Fig. 1, Table 1).

Based on retention times, fragmentation patterns and comparison with reference standards, 5α-hydroxy laxogenin (Fig. 1, structure **2**) was not detected in five of the dietary supplements (#20190, #20307-20309, #20857); diosgenin (Fig. 2, structure **4**) was the major steroidal saponin detected (Fig. 3) except for the sample #20857. Other compounds detected were Δ^4-diosgenone, glyceryl fatty acid esters, two more related diosgenin compounds and methoxyisoflavone (#20190, #20307-20309).

5α-Hydroxy laxogenin was detected in seven dietary supplements (#20310-#20313, #20315, #20833, #20834) (Fig. 3). Other diosgenin-related compounds including epoxides (Fig. 2, structures **5a**, **5b**), anzurogenin D (Fig. 2, structure **6**), diketones (Fig. 2, structures **8, 9** and diosgenin-3,6-dione,**10**) were also identified in four

supplements along with 5α-hydroxy laxogenin. In one product (#20312) arimistane, an aromatase inhibitor was also identified (Table 2), along with piperine. Recently the FDA issued a warning letter [11] citing the manufacture and sales of arimistane in several products due to false claims about the health benefits and arimistane does not meet the definition of new dietary ingredient. It is currently a World Anti-Doping Agency (WADA) prohibited substance. Paradoxically, none of the products contained laxogenin, a natural constituent of *Smilax sieboldii* [12,13].

For confirmatory purposes as well as to mimic the plausible synthetic processes (synthetic trail) associated with the compounds detected in the twelve study supplements, diosgenin was synthetically converted to closely related analogs as shown in Fig. 2. The readily available diosgenin was subjected to epoxidation with meta-perchlorobenzoic acid to yield a 3:1 mixture of both alpha and beta-5,6-epoxy derivatives (Fig. 2, structures **5a** & **5b**). The resulting mixture was subjected to acid catalyzed epoxide ring opening with aqueous methanol in the presence of a cation exchange resin, Dowex® 50 WX2, to yield anzurogenin D (Fig. 2, structure **6**). Oxidation of the anzurogenin D with Dess-Martin periodinane reagent resulted 5α-hydroxyspirostan-3,6-dione (Fig. 2, structure **8**). Similarly, the oxidation of laxogenin (Fig. 1, structure **3**) with Dess-Martin periodinane reagent resulted in diketone, chlorogenone (Fig. 2, structure **9**). The products obtained in this process served as reference standards for the unequivocal identification and confirmation of various exogenous, un-labeled spirostane-type analytes found in these supplements.

For example, analysis of the sample #20310 resulted in the identification of several un-labeled components viz., epoxyspirostan-3ols (Fig. 2, structures **5a** and **5b**), anzurogenin D (Fig. 2, structure **6**), and 5α-hydroxyspirostan-3, 6-dione (Fig. 2, structure **8**) along with claimed 5α-hydroxy laxogenin. The presence of **5a** and **5b** along with their 3:1 isomeric distribution, **6** and/or **8** in products labeled to contain 5α-hydroxy laxogenin (#20310, # 20311, #20313 and #20315) highlights the poor quality of raw materials used in these dietary supplements and suggests that the raw materials possibly originated from synthetic source.

In another set of supplement products (#20190, #20307-09) purported to contain 5α-hydroxy laxogenin, identified only diosgenin and Δ^4-diosgenones - emphasizing the lack of quality assurance and possibly misleading consumers with improper labels on these supplements. 5-Methyl-7-methoxyisoflavone, commonly referred to methoxyisoflavone, is marketed as an anabolic promoted in bodybuilding supplements [14,15]. Unclaimed methoxyisoflavone was found to be present in four of the twelve dietary supplements. Product #20857 was found to contain two unclaimed compounds, DHEA (dehydroepiandrosterone) and testosterone phenylpropionate. DHEA is WADA prohibited compound.

3.1. Unique fragmentation pattern of spirostanes – diosgenin, laxogenin and 5α-hydroxy laxogenin

In the positive ion mode, 5α-hydroxy laxogenin, laxogenin and diosgenin produced protonated $[M+H]^+$ molecular ions at *m/z* 447.3104, 431.3144 and 415.3204, respectively. The fragment ions of diosgenin were observed at *m/z* 271.2058 $[M+H\text{-}C_8H_{16}O_2]^+$ via loss of a spirostane group, and predominant ion at *m/z* 253.1953 $[M+H\text{-}C_8H_{16}O_2\text{-}H_2O]^+$due to loss of water. Other minor fragments ions with low intensities for each spirostane were listed in Table 1. The same fragmentation phenomenon was observed for both laxogenin and 5α-hydroxy laxogenins (Table 1). The unique fragmentation pattern associated with three spirostanes is outlined in Fig. 4 and could serve as a mass spectroscopic tool for the identification of un-targeted analysis of various spirostanes.

4. Conclusion

Ultra-high performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry were used for the analysis of dietary supplements claiming to contain 5α-hydroxy laxogenin. This method involved the use of $[M+H]^+$ ions in the positive ion mode withextracted-ion chromatogram (EIC). In this study, details of the analysis and identification of diosgenin and diosgenin related compounds from laxogenin containing dietary supplements being sold on the US market are described. Twelve dietary supplements were analyzed and seven of 12 supplements were found to contain 5α-hydroxy laxogenin. Five products did not contain either 5α-hydroxy laxogenin or laxogenin. Diosgenin or diosgenin analogs were detected in most of the supplements tested, highlighting the inferior quality of raw materials used in these supplements that are targeted for bodybuilding purposes. To the best of our knowledge, these ingredients should not be in dietary supplements as there was no history of safe use in foods, lack of the GRAS information as well no submission to the FDA for notification as new dietary ingredients.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This research is supported in part by Uniformed Services University of the Health Sciences (USU) grant number HU0001-16-2-0030, administered by The Henry M. Jackson Foundation for the Advancement of Military Medicine, Inc. (HJF) and "Science Based Authentication of Dietary Supplements" funded by the Food and Drug Administration, grant number 5U01FD004246-07. The authors would also like to thank Andrea Lindsey for her contributions and Prof. Jon F. Parcher for his help in correcting grammar and support. The information or content and conclusions do not necessarily represent the official position or policy of, nor should any official endorsement be inferred on the part of USU, the Department of Defense, FDA, or the U.S. Government.

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