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The pseudoendogenous anabolic steroid 1,4-androstadiene-3,17-dione does not occur naturally in *Rhodiola rosea* L. radix and rhizome

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ABSTRACT

Background: *Rhodiola rosea* L. roots and rhizome (RRR) extracts are used as dietary supplements for temporary relief of symptoms of stress, such as fatigue, sensation of weakness, and for improvement of mental and cognitive function. RRR containing products are also of interest to sports nutrition experts and antidoping authorities. It has been suggested that nutritional supplements containing *Rhodiola rosea* extracts be examined for the presence of (pseudo-) endogenous steroids, specifically for the presence of 1,4-androstadiene-3,17-dione (ADD) which can potentially lead to adverse analytical findings (AAFs) in doping controls.
Aim of the study: The aim of the present study was to detect and quantify ADD in *Rhodiola* roots and rhizomes collected in various geographical locations of North America, Europe, and Asia.
Results: ADD was not found in RRR by any of three methods used in this study. No peaks corresponding to ADD in chromatograms of RRR extract were detected at the limit of quantification corresponding to 1.6 ng/g of ADD in dry rhizomes of *Rhodiola rosea*.
Conclusion: The anabolic steroid 1,4-androstadiene-3,17-dione was not detectable in the *Rhodiola rosea* roots and rhizomes, at least not present in amounts which may be a concern for athletes who want to avoid the consumption of this anabolic agent with *Rhodiola* dietary supplementation.

1. Introduction

Rhodiola rosea L. roots and rhizome (RRR) extracts are widely used as active ingredients in adaptogenic herbal medicinal products and dietary supplements for temporary relief of symptoms of stress such as fatigue, sensation of weakness, and for improvement of mental and cognitive activity (Panossian et al., 2010; EMA, 2011; EFSA, 2017; Panossian and Wagner. 2005; Panossian and Wikman, 2009, 2014).

R. rosea may have beneficial effects on physical performance, mental performance, and certain mental health conditions (Hung et al., 2011; Lee et al., 2009; Noreen et al., 2013).It seems that the predominant effect is adaptogenic rather than ergogenic, with a better tolerance of the exercise induced stress related to enhancement of the defense response of the neuroendocrine-immune system and the decrease of the stress-induced oxidative damage (Megna et al., 2012; Ahmed et al., 2015; Hovhannisyan et al., 2015; Walker and Robergs, 2006; Ishaque et al., 2012).

Since athletes who undergo strenuous exercise, especially in endurance sports, frequently use herbal supplements to improve performance (Domene, 2013; Baker et al., 2014; Bucci, 2000), RRR-containing products are under consideration by sports nutrition experts and antidoping authorities, which control the presence of banned anabolic steroids (Kicman and Gower, 2003) in dietary supplements (Walpurgis et al., 2016). Thus, the results of a recently published study (Walpurgis et al., 2016) suggest the presence of the undeclared anabolic steroid 1,4-androstadiene-3,17-dione (*syn.* boldione, Fig. 1) in various nutritional supplements containing root or rhizome extracts of *Rhodiola rosea* L. In fact, in 3 of 9 products presumably containing RRR extracts, ADD was detected in the range of 24–312 ng/g (Walpurgis et al., 2016). The authors of this systematic study conclude that *"…the presence of (pseudo-)endogenous steroids in nutritional supplements containing R. rosea extracts has to be further investigated, as especially 1,4-androstadiene-3,17-dione can potentially lead to Adverse Analytical Findings(AAF)s in doping controls."* (Walpurgis et al., 2016). However, this conclusion is not evidence-based since the presence of *Rhodiola rosea* extracts in the supplements was not documented experimentally. Furthermore, roots

Abbreviation: ADD, 1,4-androstadiene-3,17-dione (*syn.* boldione); RRR, *Rhodiola rosea* L. roots and rhizome; TMS, trimethylsilyl

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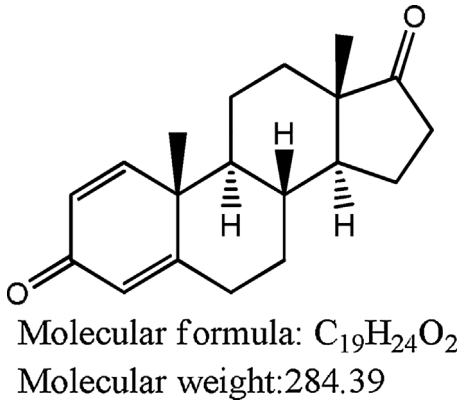

Fig. 1. Chemical structure of 1,4-androstadiene-3,17-dione.

and rhizomes of *R. rosea* have not been tested for the presence of anabolic steroids. Also, this conclusion might be misinterpreted as a warning related to the stimulating effects of roots and rhizomes of *R. rosea* due to the presence of anabolic steroids and might be considered as doping. Normally, human anabolic steroids dehydroepiandrosterone, 4-androstene-3,17-dione, and 1,4-androstadiene-3,17-dione are biosynthesized from cholesterol predominantly in mammalian adrenals and gonads, but are not common for the plant kingdom. Phytosterols can be also metabolized into 17-keto steroids by enzymes of microbial origin (Malaviya and Gomes, 2008), however the occasional presence of minor amounts of anabolic 17-keto steroids in the pollen of Scotch pine *Pinus silvestris* L. (Saden-Krehula et al., 1971) have not been well documented or confirmed, particularly their presence in the underground parts of plants have not been demonstrated to our best knowledge (Fig. 1).

Therefore, the aim of the present study was to verify the presence of an anabolic steroid, specifically ADD in *Rhodiola* roots and rhizomes collected in various geographical locations of North America, Europe, and Asia. Along with the standard analytical method used in this study for identification of ADD in the form of TMS derivatives in dietary supplements (Geyer et al., 2004; Walpurgis et al., 2016), we developed two new alternative methods of analysis of native ADD in RRR to exclude possible artifacts during derivatization and compared the results obtained by different methods – UPLC-UV (Section 2.4.1) and UHPLC/QTOF-MS (Section 2.4.2). According to regulatory guidelines, these category II (determination of limits of impurities or degradation products) and IV (identification tests) analytical methods were validated for specificity and limits of detection (LOD) (Swartz and Krull, 2012).

2. Materials and methods

2.1. Plant materials

The samples of *Rhodiola rosea* L. roots and rhizomes were collected in Norway (I, batch Nr U906F9), Canada (II, Batch Nr U90676), and Altai, Russia (III, Batch Nr 10130304). Plant material was identified as *Rhodiola rosea* L. roots and rhizome by macroscopic and microscopic examination, by HPTLC and HPLC (Supplement 1). The voucher specimens were deposited in both Institutions – (A) Shanghai Research Center for TCM Modernization, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai in China and in (B) Swedish Herbal Institute AB in Sweden. The roots of *Bryonia alba* L. (IV, Batch Nr SS.1269) were collected in Armenia.

2.2. Reference standard and solvents

1,4-Androstadiene-3,17-dione reference standard (LOT:D03N7B24210, purity: ≥98%) was purchased from Shanghai Yuanye Bio-Technology Co., Ltd (Shanghai, China) and Sigma-Aldrich and used for standard curves development. Tyrosol (purity: ≥99.5%) and cinnamyl alcohol (purity: ≥98%) were purchased from Merck KGaA, Darmstadt, Germany; salidroside, rosavin, rosarin, and rosin reference standards (purity: ≥98%) were purchased from Phytolab GmbH & Co. KG. (Vestenbergsgreuth, Germany) and used for standard curves development.

The solvents (water, methanol and acetonitrile) used for extraction and chromatography were of high performance liquid chromatographic (HPLC) grade from Waters Corporation, USA Merck, Darmstadt, Germany; ROE Scientific Inc., USA; Sinopharm Chemical Reagent Co, Ltd. Shanghai, China; High-purity nitrogen (99.9%) was used for sample preparation. Ultrapure water was prepared by a Milli-Q system (Millipore, Boston, MA, USA).

2.3. Preparation of the analytical and reference standard samples

2.3.1 0.5 g of powdered roots and rhizomes obtained from 40 g of dried RRR was macerated with 50 ml of methanol for 30 min by frequently shaking and extracted under a reflux condenser in a water bath at 80 °C for 2 h, cooled, combined, and filtered. The filtrate was evaporated to dryness; the residue was dissolved in 2 ml of methanol, filtered through 0.45 µm pore size filter and analyzed.

2.3.2 TMS ethers were prepared as described earlier (Geyer et al., 2004; Walpurgis et al., 2016).

2.3.3 Stock solution of ADD with concentration of 2 mg/ml in methanol was further diluted with methanol to the concentrations of 200 µg/ml, 20 µg/ml, 2 µg/ml, 200 ng/ml, 20 ng/ml and 2 ng/ml.

2.3.4 The trimethylsilyl derivative of the reference standard ADD was obtained by treatment with *N*-methyl-*N*-trimethylsilyltrifluoracetamide/NH_4I/ethanethiol (100:2:3, v:w:v) at 60 °C for 20 min and subjected to GC–MS analysis.

2.4. Analytical methods

The experiments were performed independently in two analytical laboratories of (A) Shanghai Research Center for TCM Modernization, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai in China and in (B) Swedish Herbal Institute AB in Sweden.

The presence of ADD in RRR roots was analyzed with various chromatographic techniques – HPLC, and GC using several detection methods including UHPLC-UV; UHPLC/QTOF-MS and GC–MS methods in lab (A), while the presence of all Rhodiola specific markers – by UPLC-UV in lab (B).

The limits of detection (LOD) and quantification (LOQ) of HPLC methods were evaluated by calculations based upon the standard deviation of the response (σ) and the slope (S) of calibration curve and the following formulas: LOD = 3.3 σ/S and LOQ = 10 σ/S. The limit of detection for the GC–MS method was estimated by assessment of signal-to-noise (S/N) ratio between 3:1 and 2:1 on the chromatogram of ADD.

Specificity, generally defined as the ability of the UPLC methods to unequivocally assess the analyte of interest in the presence of potential interferences, was evaluated in accordance with the new regulatory guideline (USP 25). In addition, to evaluate the resolution between the ADD peak and the next peak, a peak purity test based on photodiode array (PDA) detection or mass spectral detection of characteristic ions (specifically – the molecular ion of ADD with *m/z* 285) was applied to demonstrate that ADD was pure with no co-eluting impurities.

2.4.1. UPLC-UV method

2.4.1.1. Analytical instrumentation and chromatography details. Agilent 1290 infinity II System (Agilent Technologies, Palo Alto CA, USA) equipped with a high-speed pump, a vial sampler, a MCT and a DAD detector; Milli-Q Synthesis A10 System (MILLIPORE, Bedford, MA, USA); BRANSON B3500S-DTH sonicating bath (BRANSON (Shanghai) Co. Ltd., Shanghai, China) was used.

The UPLC column (Waters ACQUITY UPLCTM HSS T3 C18 column

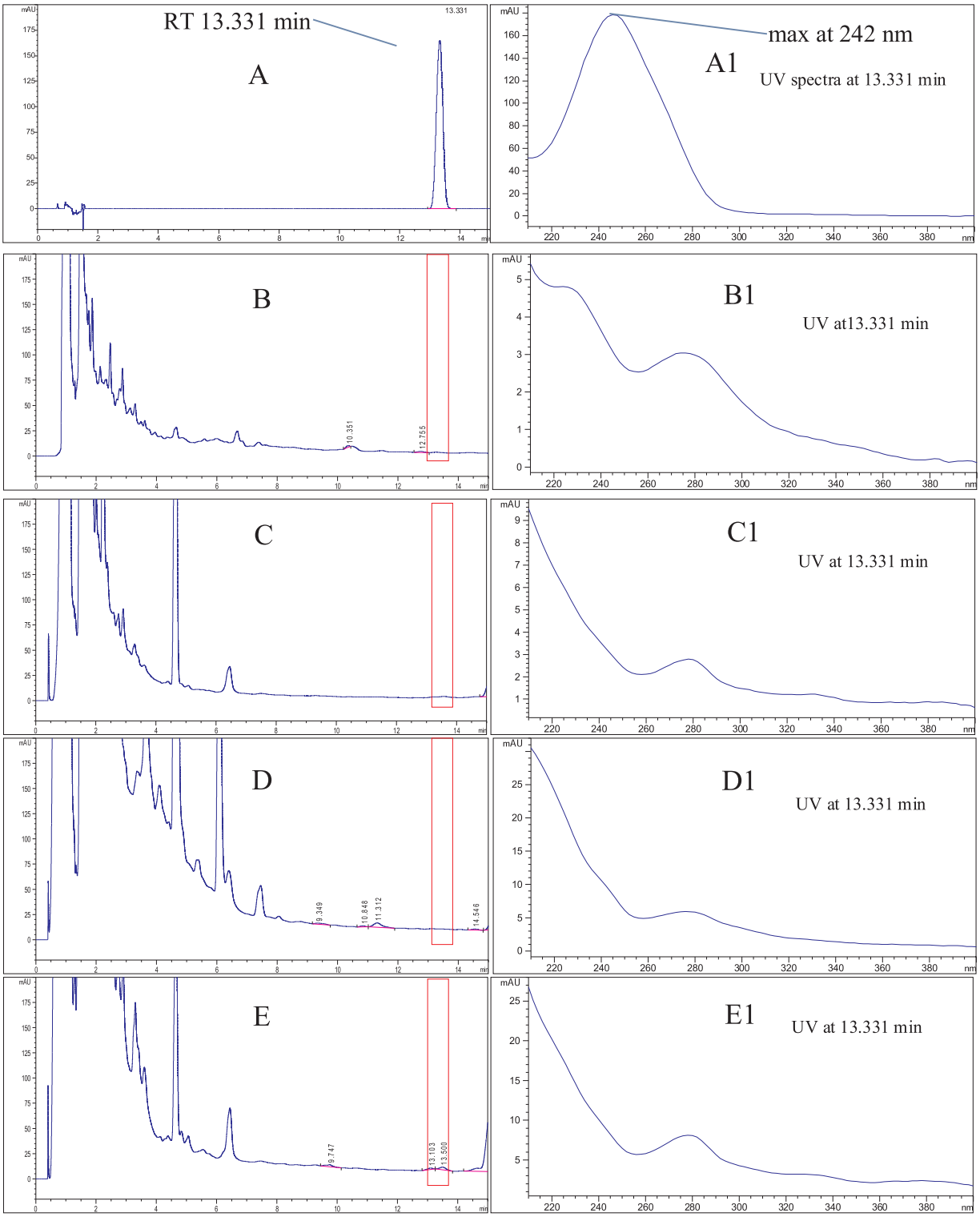

Fig. 2. UHPLC chromatogram of the reference standard ADD (A), and samples of Bryonia I (B) and Rhodiola II–IV (C–E) roots. Right panels A1–E1 shows UV spectra at 13.331 min of ADD and the samples I–IV. UV spectra of ADD with max absorption at 242 nm is incorporated in the Panels A1.

(150 mm × 2.1 mm i.d., 1.8 µm, Waters, USA)) was eluted with the solvent system containing water and acetonitrile in the ratio 65: 35, V/V. with flow rate 0.3 ml/min for 15 min at 30 °C. Injection volume 5 µl, detection at 240 nm. Test solutions are described in 2.3.1.

2.4.2. UHPLC/QTOF-MS method

2.4.2.1. Analytical instrumentation and chromatography details. Waters Xevo G2-S QTOF mass spectrometer (Waters, Manchester, UK) connected to a Waters ACQUITY UPLC system (Waters, Milford, MA, USA) equipped with a binary solvent manager, a sample manager-FL, a column manager and a PDA eλ detector via a Zpray ™ ESI source was used. Test solutions and UHPLC conditions were the same as described in 2.3.1. and 2.4.1.

Positive ion mode was utilized. ESI source parameters were set as follows: spray voltage, 2.5 kV; cone voltage, 60 V; source offset voltage, 80 V; source temperature, 100 °C; desolvation temperature, 400 °C;

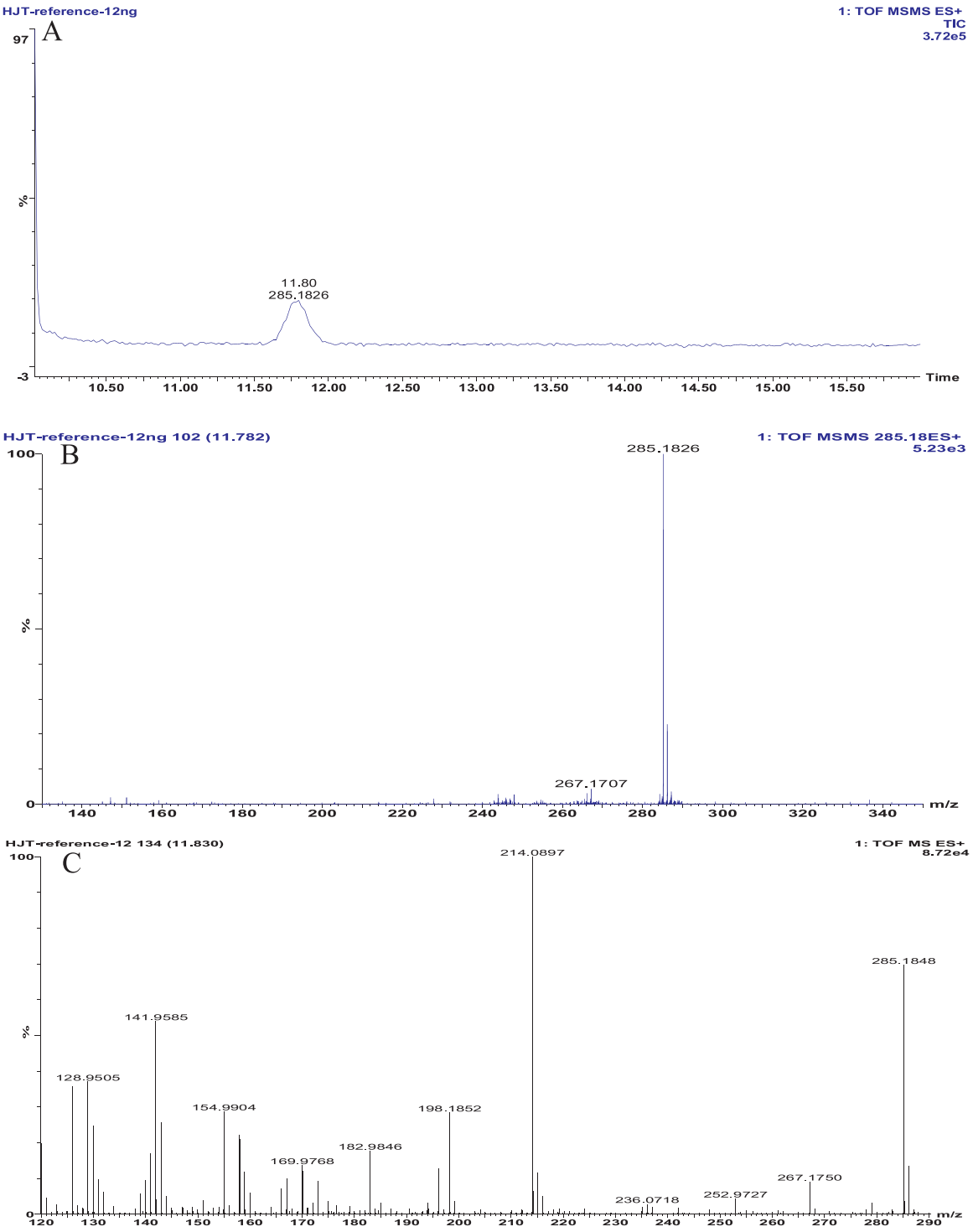

Fig. 3. Total ion chromatogram (A), mass spectrum of molecular ion (B) and mass spectrum of fragment ions of ADD (C) obtained with UHPLC/QTOF-MS.

cone gas flow rate, 40 l/h; and desolvation gas flow rate, 800 l/h. The mass analyzer scanned over a mass range of 100–700 Da in a full scan with an acquisition time of 10–16 min under the collision energy ramp of 20–60 V, and over *m/z* 100–700 for MS/MS by the same scan time. TOF MS/MS parameter was fixed: 285.18 Da.

2.4.3. GC–MS method

2.4.3.1. Analytical instrumentation and chromatography conditions. Agilent 7890A GC system coupled with 5977A MSDN-Pentane (Tokyo chemical industry Co. Ltd., Tokyo, Japan), *N*-methyl-*N*-trimethylsilyltrifluoracetamide (Tokyo chemical industry Co. Ltd, Tokyo, Japan), ethanethiol (Tokyo chemical industry Co. Ltd., Tokyo, Japan), NH_4I (AR, Sinopharm Chemical Reagent Co, Ltd. (SCRC), Shanghai, China), sodium hydroxide (AR, Sinopharm Chemical Reagent Co, Ltd. (SCRC), Shanghai, China) were used. The following conditions were applied: injection volume: 1 μl, at 290 °C; column: Agilent HP 5 MS column, 30.0 m, 0.25 mm i.d., film thickness 0.25 μm; split ratio 3:1, split flow 6 ml/min; flow rate: 2 ml/min; temperature program: 100 °C with 40 °C/min to 190 °C, with 5 °C/min to 240 °C, with 40 °C/min to 320 °C, hold 1.75 min ionization: 70 eV; electron impact (EI); the mass range: *m/z* 50–550. Test solutions are described in 2.3.2.

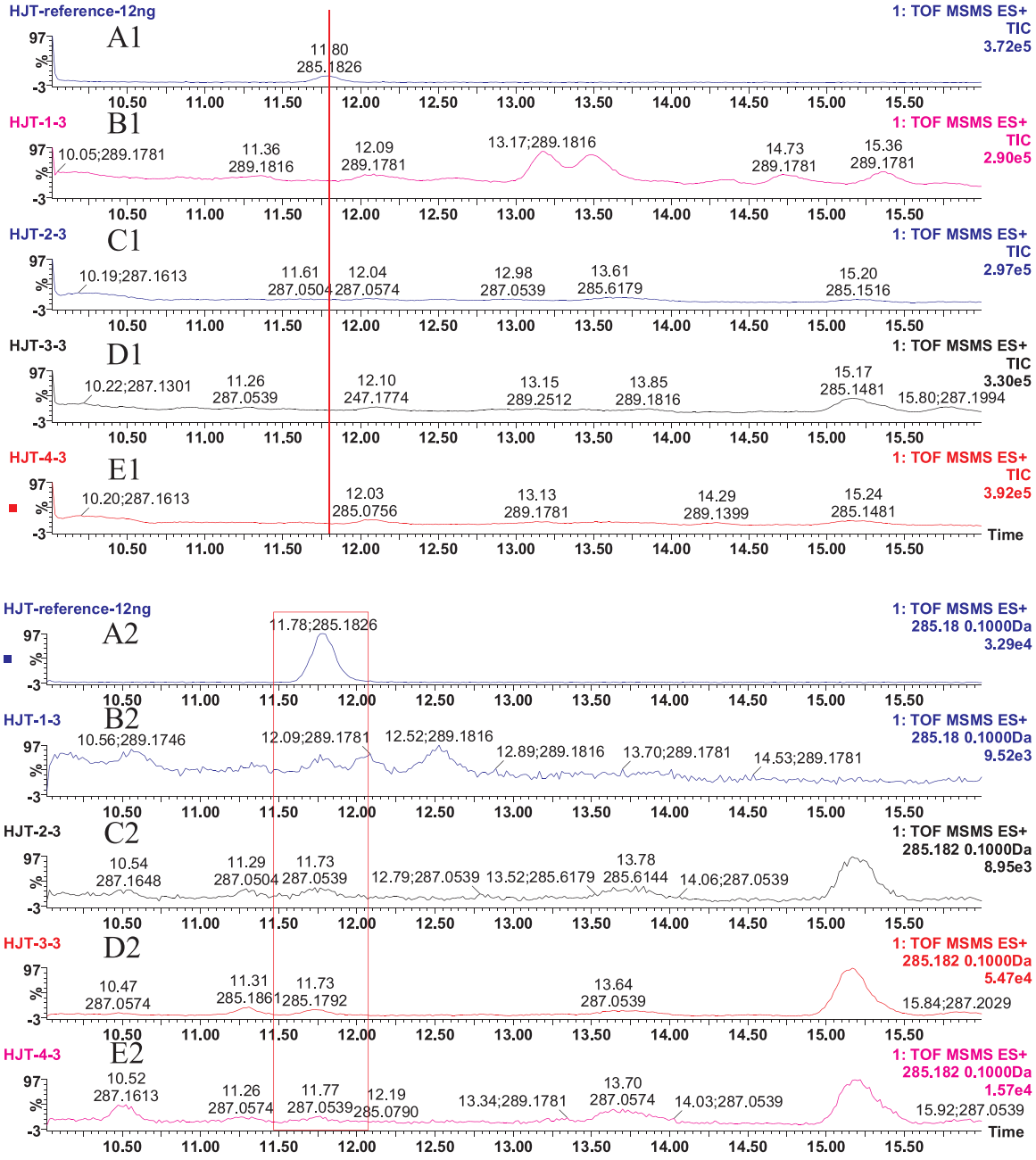

Fig. 4. Total ion chromatograms UHPLC/QTOF-MS of ADD (A1) and samples of Bryonia I (B) and Rhodiola II–IV (C-E) roots; extracted ion chromatograms of *m/z* 285.18 of ADD (A2) and samples I–IV (B2-E2).

3. Results and discussion

The standard analytical method of identification of ADD in the form of TMS derivatives in dietary supplements by GC–MS (Geyer et al., 2004; Walpurgis et al., 2016) has some disadvantages related to derivatization, e.g., some degradation products, lack of a stable and intense molecular ion, esterification of the carbonyl group in the enolic form, etc. Therefore, an analysis method for native ADD detection in the RRR samples by HPLC with the most common technique (UV-PDA) and the rather sensitive method (QTOF-MS) was developed. This procedure was simpler in preparation and the results could be unequivocally interpreted.

The content of ADD in the RRR samples was analyzed using the reference standard ADD. The UHPLC/QTOF-MS method was validated for specificity – fragment ions at *m/z* 285.1848, 267.1750, 252.9727, 236.0718, 214.0897 of the mass spectra (Fig. 3) were in accordance with reported data (Kwok et al., 2015; Chiesa et al., 2015). The limit of quantification (LOQ) of ADD was 4 ng per mL of test solution, while the signal/noise ratio was 10.97. The limit of detection of ADD was 0.4 ng per mL of test solution and corresponds to 1.6 ng/g of ADD in RRR, prepared as in 2.3.1.

The UHPLC-UV method was validated for the limits of detection (97.8 ng/ml, corresponding to 400 ng per mL of test solution of ADD in RRR, prepared as in 2.3.1.) and quantification (244.5 ng per mL of test solution), intermediate precision (RSD < 5%), and accuracy (RSD < 5%).

The GC–MS method was validated for specificity (retention time and the mass spectra of the ADD reference standard did not interfere with other constituents of the RRR sample; the molecular ion with *m/z* 285 was selected for calibration). Additionally, the limits of detection (0.61 ng per mL of test solution) and quantification (1.63 ng per mL of test solution) were also established.

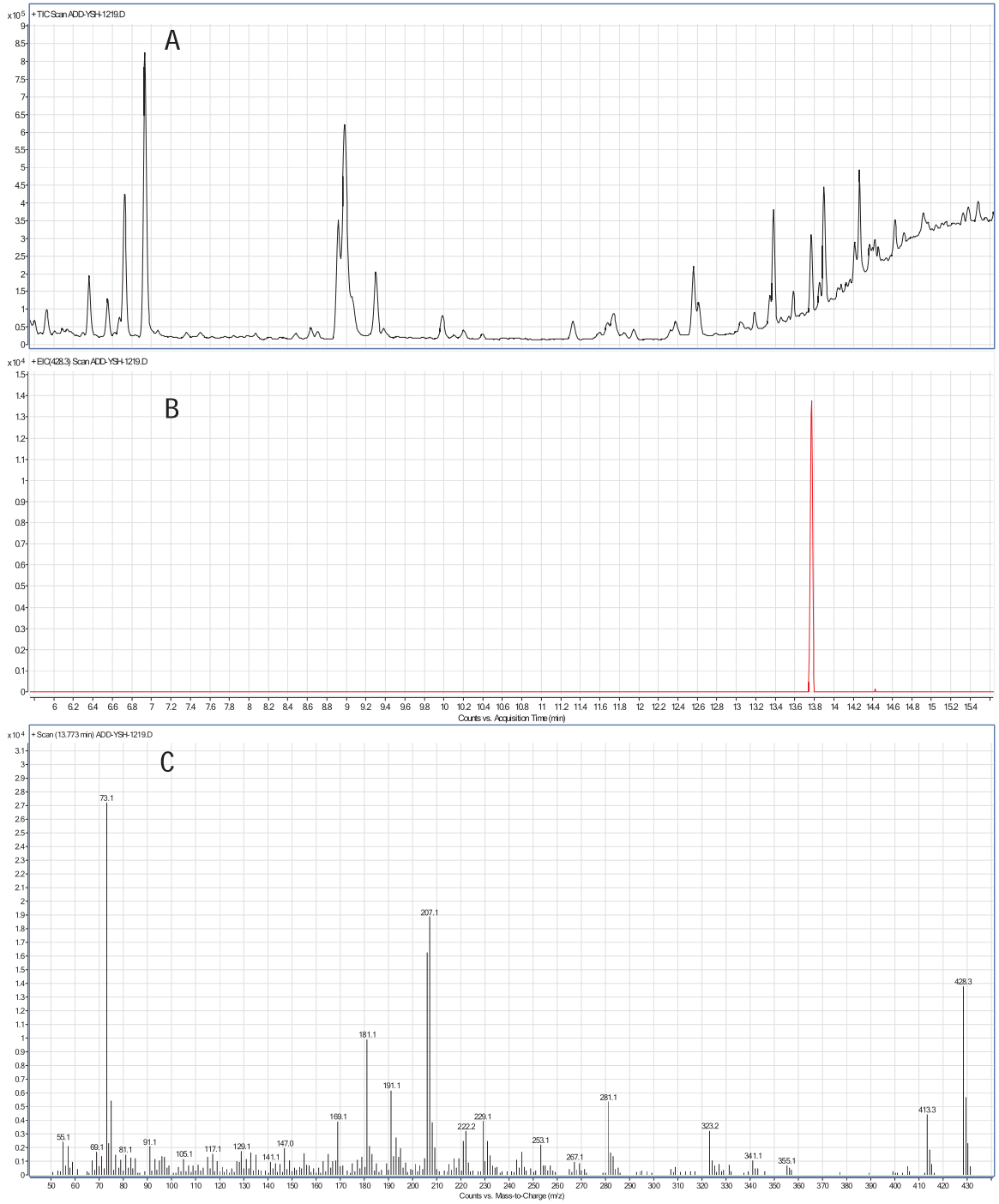

Fig. 5. Chromatogram and mass spectrum of derivatives of ADD obtained with GC–MS: total ion chromatogram (A), extracted ion chromatogram of *m/z* 428.3 (B) and spectrum at 13.773 min (C).

ADD was not found in RRR by any of the three methods used in this study (Figs. 2–6). No peak matching to ADD on chromatograms of RRR samples were detected at the limit of quantification corresponding to 1.6 ng/g and 0.8 ng/g of ADD in dry roots of *Rhodiola rosea* using the sensitive analysis methods of UHPLC-QTOF and GC–MS. Representative chromatograms are shown on Figs. 2–6.

In humans, anabolic steroids are synthesized from cholesterol (Payne and Hales, 2004). Biotransformation of phytosterols to steroid hormones has thus far not been demonstrated in either plants or humans. No evidence of the excretion of phytosterol-related anabolic steroids has been observed after consumption of phytosterol-containing functional food at the recommended dose (Verheyden et al., 2009; Ros et al., 2007). Meanwhile, microbial conversion of phytosterols to steroids has been frequently reported (Shao et al., 2015; Fernandes et al., 2003; Malaviya and Gomes, 2008), e.g., *Mycobacterium neoaurum* JC-12 isolated from soil is capable of transforming phytosterol to ADD (Shao et al., 2015). Therefore, we cannot exclude the possibility that microbial contamination of *Rhodiola rosea* L. roots and rhizome may induce biotransformation of phytosterols to ADD during the storage of roots before further processing into dietary supplements. Phytosterols were earlier identified in several *Rhodiola* species (Satsyperova et al., 1993; Tayade et al., 2013; Wang et al., 2006 Kang et al., 1992), however, data about their content in crude drug preparations are missing. The roots of *Bryonia alba* L were selected as a reference containing large amounts of

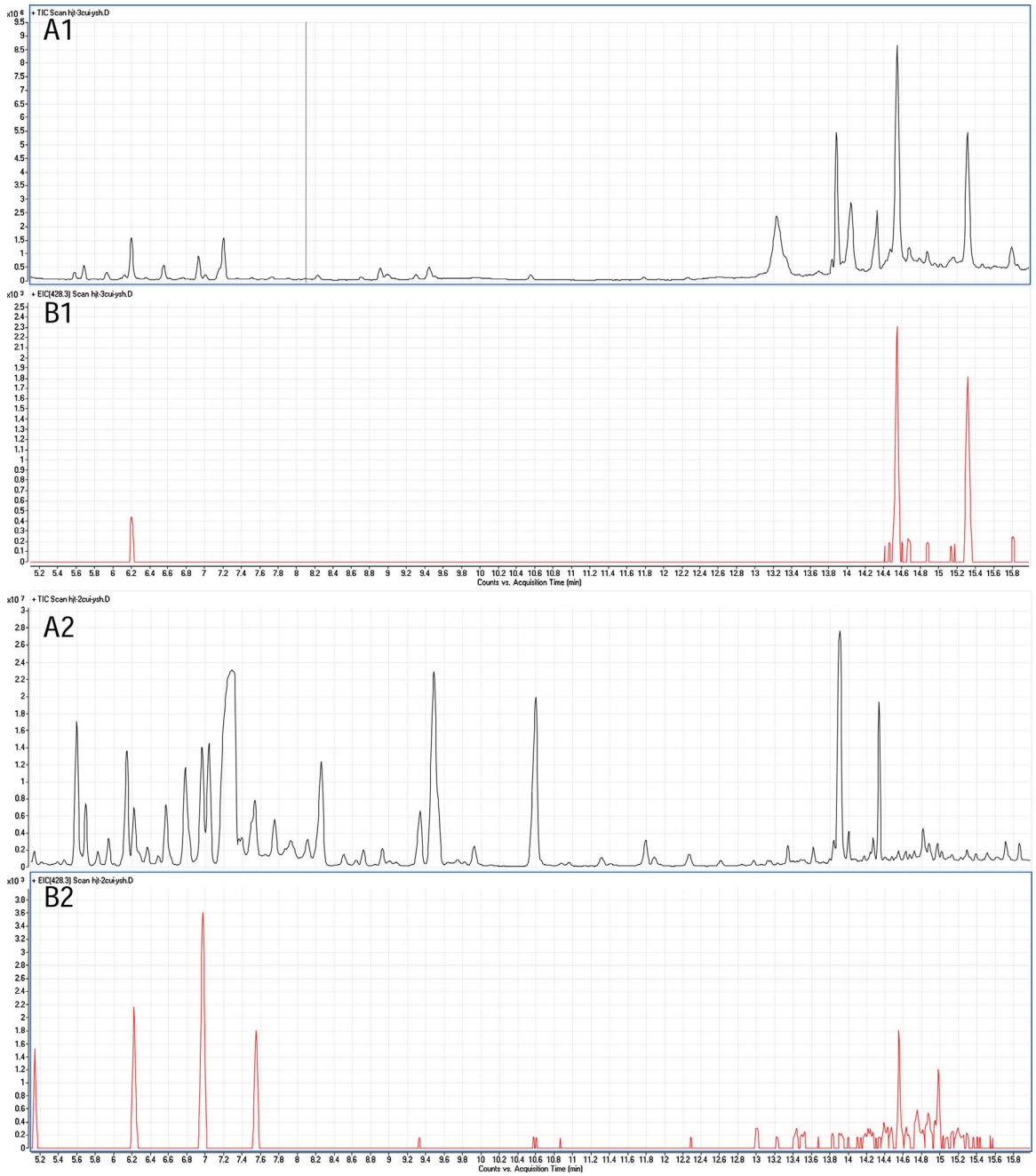

Fig. 6. GC–MS chromatograms and spectrum of derivatives of two batches of RRR samples. Total ion chromatograms (A1 and A2) and extracted ion chromatograms of *m/z* 428.3 (B1 and B2).

phytosterols (Panossian et al., 1997) sufficient for their transformation into ADD in the presence of microbial contaminations; however, the peak corresponding to ADD was not detected on the chromatograms (Figs. B and B1, B2).

The results of our study do not support the suggestion made by Walpurgis et al. (2016) with the ambitious title "Detection of endogenous and pseudoendogenous steroids in dietary supplements containing Rhodiola rosea ", that rises several questions:

– what is contribution of these anabolic steroids and overall stimulating, ergogenic and antifatigue effects of Rhodiola where salidroside and rosavins are known as active principles?

– should Rhodiola be considered as a doping method?"

The results of our study do not support the suggestion made by Walpurgis and coworkers that "*Apart from cross-contamination during production and intentional admixtures for performance-enhancing effects, unknown/novel phytosteroids as reported for Vitex agnuscastus might be also present in Rhodiola rosea*". In our study, the levels of these compounds were below the detection level. Indeed, poor quality and adulterated *R.rosea* preparations (Booker et al., 2015; Xin et al., 2015) can be products of poorly governed value chains, particularly at the early stages of supply. It has been earlier hypothesized (Booker et al., 2016) that well- controlled and well-managed value chains may prevent the occurrence of accidental or deliberate contamination and adulteration.

Regarding the presence of anabolic steroids in RRR, we can conclude that the results of our study show that ADD does not naturally occur in RRR in amounts which may induce stimulating effects in humans.

Furthermore, assuming that the underground organs of *Rhodiola rosea* indeed contains ADD in the range of 24–312 ng/g found in dietary

supplements (Walpurgis et al., 2016), we can conclude that the recommended daily dose of *Rhodiola* native extract (300 mg) contains only 8–100 ng of ADD. That is 10 −150 million-fold less of the daily anabolic dose (800–1500 mg/day) of 1,4-androstadiene-3,17-dione (Anon, 2017; Powers, 2002). Moreover, taking into account that oral bioavailability of ADD is 8%, the maximal concentration of ADD in urine will be below the WADA estimated lower limit of 5 ng/ml (WADA, 2015, 2017) and below the level of endogenous ADD, which is known to be sporadically produced at concentrations ranging from 0.751 ng mL^{-1} to 1.73 ng mL^{-1} (Verheyden et al., 2009). Therefore, it is very unlikely, that the antifatigue effect of dietary supplements containing *Rhodiola rosea* is associated with ADD. Consequently, Rhodiola intake cannot be considered as a doping method.

4. Conclusion

Anabolic steroid 1,4-androstadiene-3,17-dione is not detectable in *Rhodiola rosea* roots and rhizome, at least not in amounts which may cause concern for athletes who want to avoid the consumption of this anabolic steroid.

Conflict of interest

The authors, JD, LY, WYW, DAG declare no direct conflicts of interest. AGP is currently an independent contractor at EuroPharmaUSA Inc.

Acknowledgments

All authors contributed to the writing of the manuscript. AGP and DAG developed the scope and prepared the draft of the paper. JD, LY, WYW and AGP provided the chemistry components and figures. DAG and AGP provided critical input into the interpretation of the findings and prepared of the final draft. This work was supported by Shanghai Research Center for TCM Modernization at the Shanghai Institute of Materia Medica, Chinese Academy of Sciences, China and Swedish Herbal Institute Research and Development, Kovlingevagen 21, 31250 Vallberga, Sweden

AGP was an employee of Swedish Herbal Institute at the beginning of the study and currently is an independent consultant at EuroPharmaUSA Inc.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.phytol.2017.12.007.

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Supplement 1

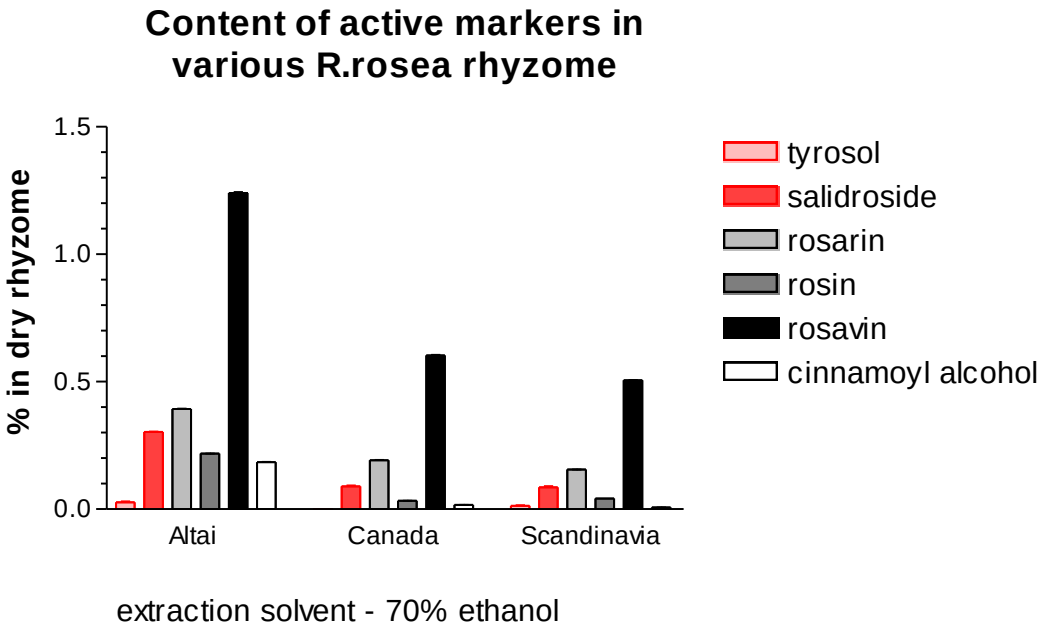

Figure 1. Content of active markers (%) in dry rhizome and roots of R.rosea from Russia (Altai), Canada (Alberta) and Scandinavia. Extraction solvent – 70% ethanol.

	Tyrosol	Salidroside	Rosarin	Rosin	Rosavin	Cinnamoyl alcohol
Altai	0.03	0.30	0.39	0.22	1.24	0.18
Canada	0.00	0.09	0.19	0.03	0.60	0.02
Scandinavia	0.01	0.09	0.16	0.04	0.51	0.01

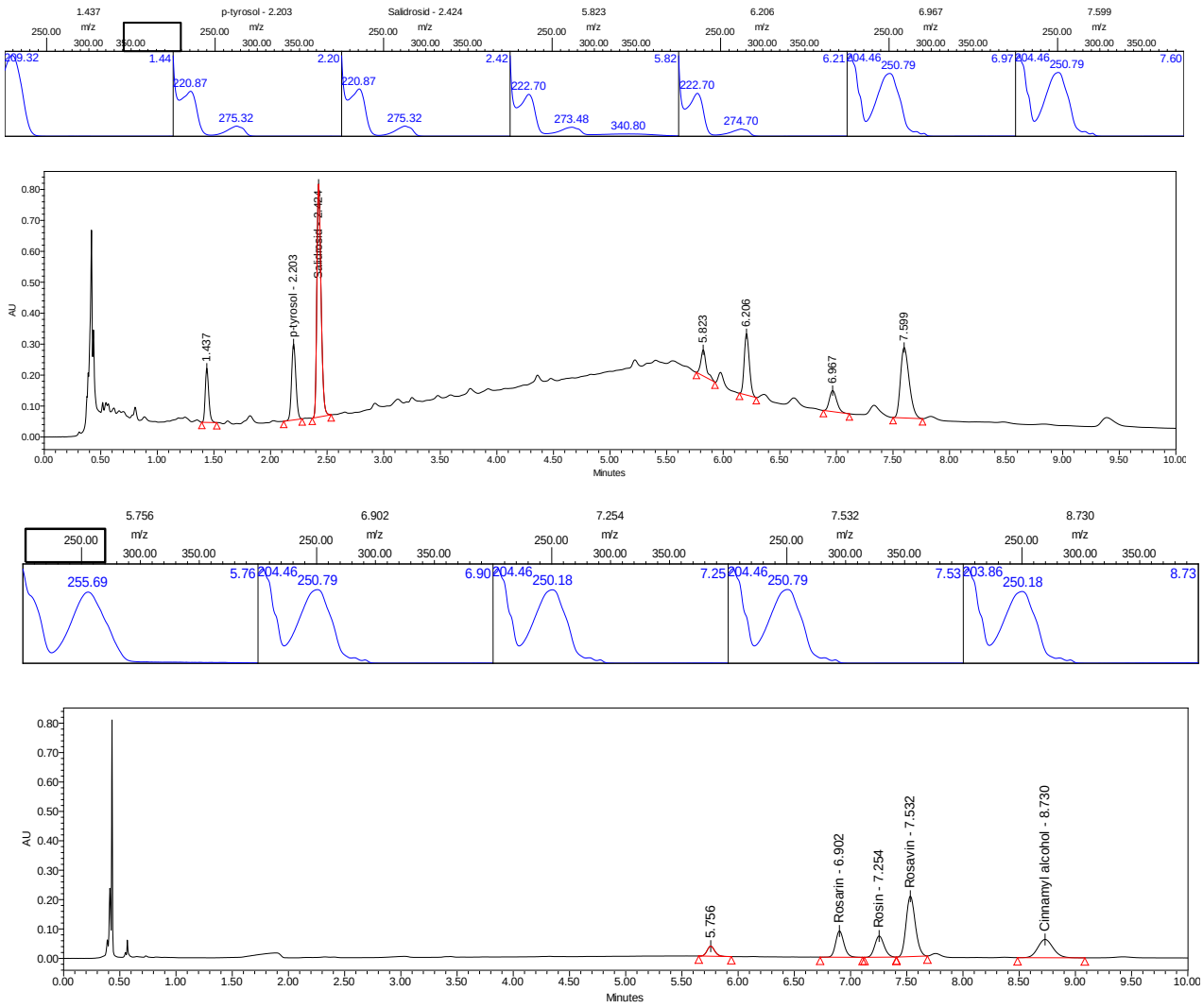

Figure 2. HPLC-UV fingerprint of R.rosea rhizome test solution: upper panel - detection at 221 nm, lower panel – detection at 252 nm. The UPLC column (Waters ACQUITY UPLC ® BEH C18, 2.1 x 100 mm) packed with octadecyl silica (1.7 µm) was eluted with the solvent system containing gradually increasing concentration (from 2.5 to 100%) of acetonitrile in water flow rate 0.6 ml/min at 70 °C.The analytical method was validated for specificity, intermediate precision and accuracy.

W. SCHÄNZER
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H. GEYER
U. MARECK
(EDITORS)

RECENT ADVANCES IN DOPING ANALYSIS (24)

Proceedings of the
Manfred Donike Workshop
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Detection of endogenous and pseudoendogenous steroids in dietary supplements containing Rhodiola rosea

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Abstract

Rhodiola rosea is a perennial plant in the *Crassulaceae* family, which grows primarily on mountains and sea cliffs in the Holarctic area. For centuries, root and rhizome extracts have been used as herbal medicine in Russia, Scandinavia, and Asia, as they are supposed to have both stimulating and adaptogenic effects. A variety of preparations containing *Rhodiola* extracts are sold worldwide as dietary supplements. As several studies report performance-enhancing effects due to an increased exercise capacity and fatigue resistance, these products are also very attractive for athletes.
From 2013 until 2015, different nutritional supplements containing root or rhizome extracts of *Rhodiola rosea* were tested for the presence of performance-enhancing drugs by means of GC-MS and LC-MS. A total of 14 products was found to contain significant amounts of the endogenous steroids 4-androstene-3,17-dione and dehydroepiandrosterone (DHEA) and the pseudoendogenous steroid 1,4-androstadiene-3,17-dione. Although the chemical composition of *Rhodiola rosea* was extensively studied in the past, there is currently no evidence for the occurrence of anabolic androgenic steroids. Consequently, the detection of several (pseudo-) endogenous steroids in dietary supplements demonstrates that the use of nutritional supplements by athletes remains a concern as preparations of dubious quality or unknown composition can potentially lead to positive results in doping tests.

Introduction

Rhodiola rosea, also known as "arctic root" or "golden root", is a perennial flowering plant belonging to the family *Crassulaceae*, which can be primarily found on sea cliffs and mountains in the holarctic area [1,2]. Due to the putative stimulating and adaptogenic effects, root and rhizome extracts have been used for centuries as herbal medicine in Russia, Scandinavia, and Asia. Moreover, numerous preparations of *Rhodiola* extracts are marketed worldwide as dietary supplements. As several studies discuss potential performance-enhancing effects due to an increased exercise capacity and fatigue resistance [1-5], products made from *Rhodiola rosea* are very attractive supplements for athletes.

Experimental

Between 2013 and 2015, a variety of nutritional supplements containing extracts from *Rhodiola rosea* was tested for adulterations and contaminations with doping agents by using GC-MS and LC-MS approaches. For the determination of anabolic-androgenic steroids, products were analyzed according to the procedure described by Geyer *et al.* [6]. In brief, 1 g of the homogenized supplement was extracted with 5 mL of methanol. Following evaporation, the dried residue was resolved in 5 mL of 0.1 M sodium hydroxide and re-extracted with 5 mL of n-pentane. The n-pentane layer was subsequently transferred to a new test tube, extracted with 2 mL of a methanol/water solution (95:5 *v:v*) and discarded. The remaining methanolic phase was evaporated to dryness, derivatized with N-methyl-N-trimethylsilyltrifluoracetamide/NH_4I/ethanethiol (100:2:3 *v:w:w*) and finally subjected to GC-MS analysis on a TSQ 8000 Triple-Quad GC-MS/MS (Thermo Fisher) coupled to a Trace 1310 gas chromatograph (Thermo Fisher).

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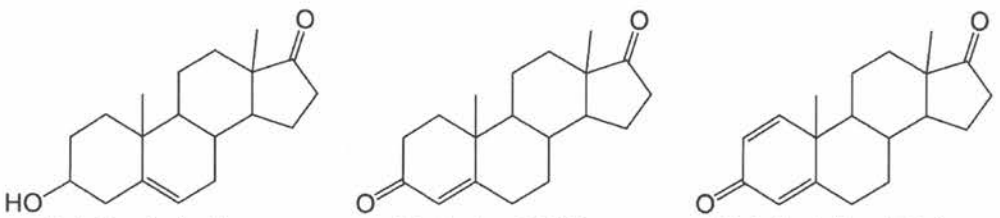
Results and Discussion

As shown in Table 1, a total of 14 products was found to contain the (pseudo-) endogenous steroids 4-androstene-3,17-dione, dehydroepiandrosterone (DHEA), and 1,4-androstadiene-3,17-dione.

#	Advertisement	Main ingredient(s)	Identified anabolic-androgenic steroid(s)	Estimated concentration
1	-	*Rhodiola rosea* extract	Dehydroepiandrosterone	44 ng/g
2	Regeneration	*Rhodiola rosea* extract, Astaxanthin	1,4-Androstadiene-3,17-dione	36 ng/g
3	-	*Rhodiola rosea* extract	1,4-Androstadiene-3,17-dione	24 ng/g
			4-Androstene-3,17-dione	< 10 ng/g
			Dehydroepiandrosterone	< 10 ng/g
4	-	*Rhodiola rosea* extract	4-Androstene-3,17-dione	43 ng/g
			Dehydroepiandrosterone	13 ng/g
5	Body weight management	*Rhodiola rosea* extract	1,4-Androstadiene-3,17-dione	54 ng/g
6	Polyphenol supplementation	Resveratrol extract, Genistein, Quercetin	4-Androstene-3,17-dione	25 ng/g
7	Polyphenol supplementation	Resveratrol extract, Genistein, Quercetin	Dehydroepiandrosterone	8737 ng/g
8	Polyphenol supplementation	Resveratrol extract, Genistein, Quercetin	Dehydroepiandrosterone	25 ng/g
9	Polyphenol supplementation	Resveratrol extract, Genistein, Quercetin	Dehydroepiandrosterone	57 ng/g
10	Advanced ATP energy	*Cordyceps sinensis*	4-Androstene-3,17-dione	< 10 ng/g
			1,4-Androstadiene-3,17-dione	< 10 ng/g
11	-	*Rhodiola rosea* extract	4-Androstene-3,17-dione	160 ng/g
			Dehydroepiandrosterone	26 ng/g
12	-	*Rhodiola rosea* extract	4-Androstene-3,17-dione	49 ng/g
13	-	*Rhodiola rosea* extract	1,4-Androstadiene-3,17-dione	312 ng/g
			4-Androstene-3,17-dione	93 ng/g
			Dehydroepiandrosterone	32 ng/g
14	-	*Rhodiola rosea*, *Menthae folium*, *Coriandri fructus*, *Curcumae* rhizoma, *Foeniculum*, and *Galangae* rhizoma extracts	1,4-Androstadiene-3,17-dione	40 ng/g

Table 1: Nutritional supplements containing extracts from Rhodiola rosea

The chemical structures of the identified analytes are displayed in Figure 1 and an exemplary full MS/MS spectrum at *m/z* 428.3 of 1,4-androstadiene-3,17-dione (bis-TMS derivative) is shown in Figure 2.



Dehydroepiandrosterone
MF: $C_{19}H_{28}O_2$,
MW: 288.21 Da

4-Androstene-3,17-dione
MF: $C_{19}H_{26}O_2$,
MW: 286.19 Da

1,4-Androstadiene-3,17-dione
MF: $C_{19}H_{24}O_2$,
MW: 284.18 Da

Figure 1: Chemical structures of dehydroepiandrosterone, 4-androstene-3,17-dione, and 1,4-androstadiene-3,17-dione.

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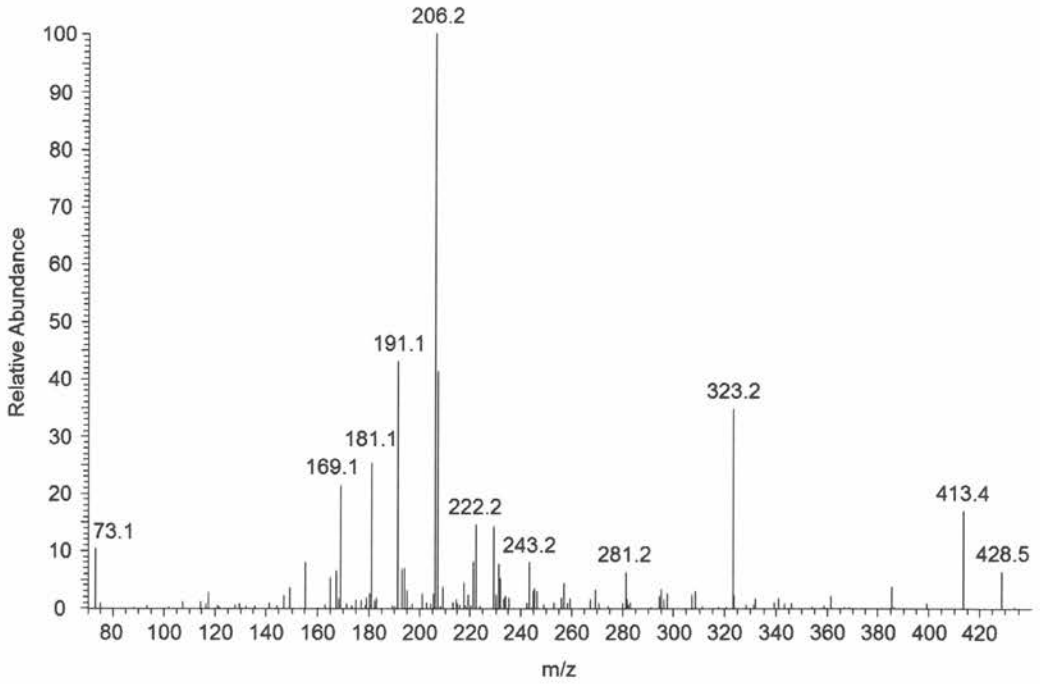

Figure 2: Full ms2 spectrum @ m/z 428.3 of 1,4-androstadiene-3,17-dione (bis-TMS derivative).

1,4-Androstadiene-3,17-dione (boldione) is a prohormone of the (pseudo-)endogenous steroid boldenone [7]. According to the WADA Technical Document TD2016 IRMS [8], even urinary concentrations below 5 ng/mL can be considered as Adverse Analytical Finding (AAF), if the results of GC/C/IRMS can unambiguously prove the exogenous origin of the substance. Consequently, athletes are advised that the ingestion of products containing low levels of boldenone prohormones in the range of ng/g could potentially cause AAFs in sports drug testing. By contrast, such effects are rather unlikely for the testosterone prohormones 4-androstene-3,17-dione and DHEA [9].

The chemical composition of *Rhodiola rosea* was extensively studied in the past and more than 140 different ingredients such as phenylpropanoids, phenylethanol derivatives, flavonoids, monoterpenes, triterpenes, and phenolic acids were identified from roots and rhizome [1,2]. However, no anabolic-androgenic steroids were found.
There are several possible explanations for the presence of undeclared 1,4-androstadiene-3,17-dione, 4-androstene-3,17-dione, and DHEA in the tested supplements. Apart from cross-contamination during production and intentional admixtures for performance-enhancing effects, unknown/novel phytosteroids as reported for *Vitex agnus-castus* [10] might be also present in *Rhodiola rosea*.

Conclusions

The presence of undeclared (pseudo-)endogenous steroids in nutritional supplements containing *Rhodiola rosea* extracts has to be further investigated, as especially 1,4-androstadiene-3,17-dione can potentially lead to AAFs in doping controls.

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