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ORIGINAL RESEARCH

Analysis of iridoids from *Harpagophytum* and eleutherosides from *Eleutherococcus senticosus* in horse urine

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ABSTRACT: LC/ESI-MS^n methods have been previously set up to detect the administration of (i) *Harpagophytum* and (ii) preparations containing a plant capable of anti-stress properties: *Eleutherococcus senticosus*. Harpagoside has been found to be the main indicator of *Harpagophytum* administration in the horse. These methods have been applied to a large number of horse urine samples of various origins. Regarding the detection of *Harpagophytum* administration, harpagoside, harpagide and 8-*para*-coumaroyl harpagide were detected together in only one sample out of 317. Eleutheroside E was found to be the main indicator of *Eleutherococcus senticosus* administration. It was detected in post-administration samples collected from two horses having received a feed supplement containing *Eleutherococcus senticosus* for several days. Out of the 382 samples tested, eleutheroside E was found in an unexpected large number of urine samples (39%) of various origins and its presence cannot be only due to the sole use of herbal dietary supplements. Copyright © 2008 John Wiley & Sons, Ltd.

KEYWORDS: *Harpagophytum*; eleutheroside; HPLC/ESI-MS^n; horse urine; population study

INTRODUCTION

Harpagophytum procumbens or *zeyheri*, also called Windhoek's root or Devil's claw, is a medicinal plant from South Africa, Namibia and Botswana, introduced to Europe a long time ago. Herbal preparations containing *Harpagophytum* with other plants are used for prevention of inflammatory processes in competition horses and in the treatment of animals suffering from lameness. The European Scientific Cooperative On Phytotherapy (ESCOP) monographs summarize the pharmacological and clinical evidence behind the therapeutic indications for the individual plant materials. ESCOP (2003) recommends the use of Devil's claw for symptomatic treatment of painful osteoarthritis, relief of low back pain, loss of appetite and dyspepsia. The effectiveness of *Harpagophytum* in the treatment of low back pain has been extensively studied (Chrubasik, 2004; Chrubasik *et al.*, 2005). Owing to its action on the muscular system, it is recommended in the treatment of chronic rheumatisms, tendonitis, osteoarthritis and arthritis (Chantre *et al.*, 2000). According to a recent review from Grant *et al.* (2007), *Harpagophytum* has significant effects on numerous pro-inflammatory markers. Consequently, it was necessary to set up a method to detect the main indicators of *Harpagophytum* administration. Apart from recent studies such as those of Seger *et al.* (2005), Baranska *et al.* (2005) and Feng *et al.* (2006), there was very little information in the literature on the analysis and extraction of these compounds from vegetal media and biological fluids. An LC/MS^n method was developed at the LCH to detect harpagoside (HS), harpagide (HG) and 8-*para*-coumaroyl harpagide (8PCHG), which are the major constituents of *Harpagophytum procumbens* and *zeyheri* (Colas *et al.*, 2006, 2008). This method was modified to detect eleutheroside E (EE) in equine urine samples. Chemical structures of HG, HS, 8PCHG and EE are displayed in Fig. 1.

EE is present in plants such as *Eleutherococcus senticosus*, also called Siberian Ginseng. This plant improves appetite and immune response; it is also helpful to relieve tiredness and stress and to improve general welfare. The improvement of physical capacity seems to be due to its action on the central nervous system and endocrine glands. *Eleutherococcus senticosus* is a major constituent of feed supplements recommended to

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Abbreviations used: EE, Eleutheroside E; ESCOP, European Scientific Cooperative On Phytotherapy; HG, harpagide; HL, hydroxylidocaine; HS, harpagoside; 8PCHG, 8-*para*-coumaroyl harpagide.

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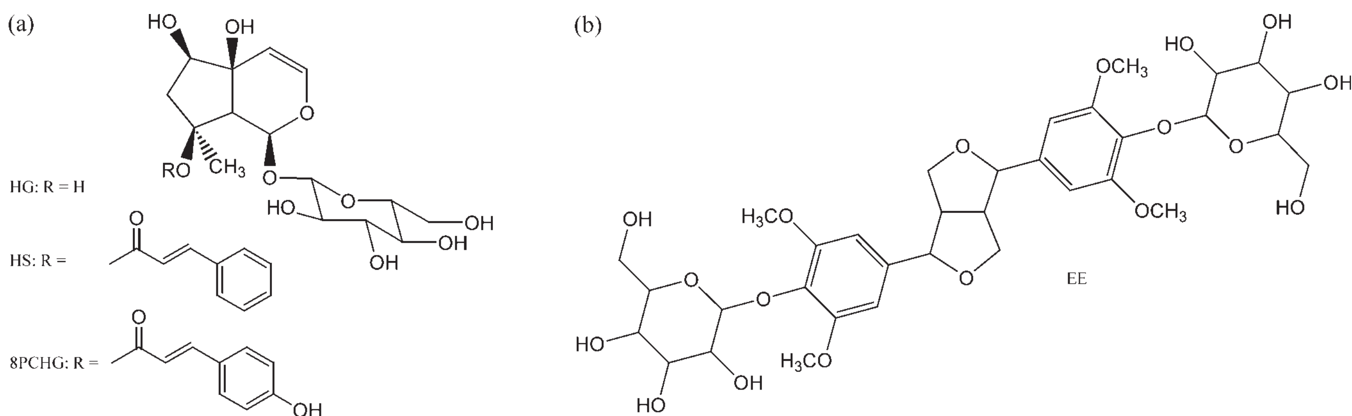

Figure 1. (a) Chemical structures of harpagide (HG), harpagoside (HS) and 8-*para*-coumaroyl harpagide (8PCHG); (b) chemical structure of eleutheroside E (EE).

improve resistance to stress of competition horses. In order to evaluate the use of nutritional preparations containing *harpagophytum*, the method was tested on post-competition samples. Furthermore, to test the efficiency of EE analysis for showing evidence of the administration of feed supplements containing EE, the method which had been previously developed was applied to the analysis of a large number of field horse urine samples and to *Eleutherococcus senticosus* post-administration samples. This paper presents the results obtained from these two surveys.

EXPERIMENTAL

Chemicals

HPLC-grade water was obtained by purifying water in a Milli-Q filtration system (Millipore, Bedford, MA, USA), HPLC-grade methanol, *n*-hexane and acetonitrile were obtained from Carlo Erba (Milan, Italy), isopropyl alcohol, formic acid, sodium dihydrogen phosphate and di-sodium hydrogen phosphate from VWR (Paris, France) and methylene chloride from JT Baker (Deventer, The Netherlands).

AbsElut Nexus (60 mg), BondElut PPL (200 mg) and BondElut C_{18} HF (500 mg) 3 mL cartridges for solid-phase extraction (SPE) were purchased from Varian (Palo Alto, CA, USA). HS was supplied by Extrasynthese (Genay, France), HG and 8PCHG were purchased from Phytolab (Hamburg, Germany) and EE from LGC Promochem (Molsheim, France). Hydroxylidocaine (HL), used as the internal standard for LC/MS quantitation, was synthesized at our laboratory. Stock solutions of standards were prepared by dissolving the selected analytes in methanol and stored at 4°C before being used.

Instrumentation

Analyses were performed on an LTQ (Thermo Electron, San Jose, CA, USA) except those of HS in routine samples, which were carried out on an LCQ Deca XP Max (Thermo Electron).

LC/MS analysis of HS, HG, 8PCHG and EE with the LTQ mass spectrometer. Analyses were performed using an Agilent 1100 Series Binary Pump, equipped with a standard autosampler, a vacuum degasser and a thermostated column compartment (Agilent, Palo Alto, CA, USA). The column used was a Zorbax Eclipse XDB-C_{18} Solvent Saver Plus (3.5 µm, 150 × 3.0 mm), fitted with a Zorbax Eclipse XDB-C_{18} (5 µm, 12.5 × 4.6 mm) analytical guard column (Agilent Technologies, Massy, France) and a 0.5 µm high-pressure frit. The mobile phase consisted of 0.1% formic acid in water (A solvent), and methanol (B solvent). The composition was 90:10 (A:B) from 0 to 2 min, 40:60 (A:B) at 4 min, 20:80 (A:B) at 5 min and (0:100) (A:B) from 10 min up to 15 min, with column re-equilibration from 16 to 20 min at 10% B. The flow rate was set to 400 µL/min. Aliquots of 10 µL of sample were injected.

The LTQ was operated in the ESI positive mode (+4.5 kV). The flows of sheath and auxiliary gases were set at 40 and 5 arbitrary units, respectively. The capillary temperature was maintained at 350°C. The q_z factor was set to 0.25 for all MS^n experiments. For analyte identification, the product ions resulting from collision-induced dissociation (CID) of $[M + Na]^+$ pseudomolecular ions were monitored in the MS^2 or MS^3 mode with the following *m/z* scanning: *m/z* 387.1 → [200–390] for HG, *m/z* 517.1 → *m/z* 369.1 → [200–380] for HS and *m/z* 533.1 → *m/z* 369.1 → [200–380] for 8PCHG. The consecutive reaction monitoring (CRM) mode was used for quantitation, using the following transitions: *m/z* 517.1 → *m/z* 369.1 → *m/z* 351.1 for HS and *m/z* 533.1 → *m/z* 369.1 → *m/z* 351.1 for 8PCHG. HG quantitation was done in full-scan mode.

EE quantitation was performed in the ESI positive mode with selected reaction monitoring (SRM) detection. CID was carried out on $[M + Na]^+$ ions and the transition *m/z* 765.2 → *m/z* 603.2 was monitored. For EE characterization, the LTQ was operated in the ESI negative mode and the product ions resulting from CID of $[M + HCO_2]^-$ ions were monitored in MS^2 with the following *m/z* scanning: *m/z* 787.1 → [215–800].

In all cases, HL was detected in the SRM mode on the *m/z* 251.1 → *m/z* 86.0 transition.

LC/MS analysis of HS with the LCQ Deca XP Max mass spectrometer. The liquid chromatography system consisted of a Surveyor MS Pump equipped with a surveyor autosampler

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(Thermo Electron). The column and precolumn were those described above. The flow rate was set to 300 μL/min. The LCQ Deca XP Max was operated in the ESI positive mode. Both flows of sheath and auxiliary gases were set at 20 arbitrary units for HL; they were set at 30 and 7 arbitrary units, respectively, for HS. The capillary temperature was maintained at 300°C. The q_z factor was set to 0.25 for all MS^n experiments. The CID of $[M + Na]^+$ ions was operated both in the MS^2 and in the MS^3 mode with *m/z* scanning of daughter ions, *m/z* 517.2 → [350–520] and *m/z* 517.2 → *m/z* 369.1 → [200–380] for HS.

Eleutherosides animal experiment

Two doses of Twydil® Hippacan+C, i.e. 70 mg of EE, were given orally once a day for 5 days to two thoroughbred adult horses weighing 450–500 kg. A urine sample was collected before the beginning of the treatment. For the first horse, three urine samples were collected 4 h, 12 h and 24 h after the last administration. For the second horse, seven urine samples were collected from 2 to 24 h after the last administration and one urine sample was collected each day up to 5 days after administration.

Analysis of post-race negative urine routine samples. For HS, 317 post-competition urine samples collected from 123 harness race horses, 140 thoroughbred race horses, 48 sport horses and six unknown horses were tested. For EE, 382 urine samples collected from horses after competition, at training or from non-training horses were tested. The population of post-competition horses was harness race horses n = 133, thoroughbred race horses n = 74, and sport horses n = 77. Twenty-two urine samples were collected from horses at training and 23 urine samples were collected from out-of-competition sport horses. In addition, horses of unknown origins (n = 53) were also tested.

Sample preparation methods

Extraction of HS, HG and 8PCHG from urine samples. One milliliter of urine was diluted with 2.5 mL of 0.25 M phosphate buffer pH 7.7 and 6.5 mL of water, and 2.5 ng of internal standard HL were added. pH was controlled and adjusted to 7.7 if necessary. The sample was then centrifuged 30 min at 3000***g*** before performing SPE. HS and 8PCHG were extracted with a Rapid Trace SPE workstation (Zymark, Hopkinton, MA, USA), using AbsElut Nexus cartridges successively rinsed with water, a 70:30 water–methanol mixture and *n*-hexane. Analytes were eluted with a 80:20 methylene chloride/isopropyl alcohol mixture (Colas *et al.*, 2008). HG was extracted in two steps: urine prepared as described above was extracted on a BondElut PPL cartridge. After rinsing with water (3 mL), elution was carried out with a 50:50 acetonitrile–methanol mixture. The solution eluted from this cartridge was evaporated and then dissolved in 100 μL of methanol and 3 mL of water. This solution was extracted on a BondElut C_{18} HF cartridge, successively rinsed with *n*-hexane and with a 70:30 water–methanol mixture. Elution was performed with a 50:50 water–methanol mixture (Colas *et al.*, 2008). After extraction, samples were evaporated to dryness at 48°C with a TurboVap LV Evaporator (Zymark) for 15–20 min. The dry residue was dissolved in 25 μL of methanol and 175 μL of water before analysis by LC/MS^n.

Extraction of EE from urine samples. The analytical procedure described before for HS and 8PCHG extraction was applied for the extraction of eleutheroside E in urine. The only modification involves the rinsing of the cartridge that was carried out using water, a 95:5 water–methanol mixture and then *n*-hexane.

RESULTS AND DISCUSSION

Previous results from *Harpagophytum* study

The sample preparation procedures described above provided recovery yields of 85 and 77%, for HS and 8PCHG, respectively (with RSD determined between 7 and 19% on 12 urine samples spiked at 2 and 20 ng/mL), with the extraction recoveries obtained at 2 and 20 ng/mL being similar. For HG, the mean value of recovery yield was 75% (RSD between 14 and 19%; Colas *et al.*, 2008).

The possibilities offered by the different mass spectrometers available at the LCH were tested in previous work; the LTQ mass spectrometer was the most efficient for qualitative and quantitative analysis so that a large part of the study was performed on this instrument. For qualitative purposes, analyses were carried out in the full-scan MS^2 or MS^3 mode. Extracted ion chromatograms obtained from a solution of HS at 100 pg/μL in MS^3 are presented with the mass spectrum of HS on Fig. 2.

HS is the main indicator of *Harpagophytum* administration. HG can be detected in post-administration urine samples a few hours after the administration of *Harpagophytum* preparations and 8PCHG from 3 to 10 h after administration. Owing to a strong matrix effect observed in urine, quantitation of HS in post-administration samples was done using the standard addition method as previously described (Colas *et al.*, 2006). As a result, when HS, HG or 8PCHG were detected in a post-race sample, their concentrations were estimated using a spiked sample.

Detection of HS in post-race samples

The samples selected for this study were found to be ‘negative’ by the routine testing procedures employed at the LCH. Using the extraction method described above, eight of the 317 horses tested (Table 1) were found to contain HS. In six samples out of eight, HS was the only indicator of *Harpagophytum* administration detected in these samples. HS concentration was about 1 ng/mL. In one sample, HS was concomitantly detected with 8PCHG. In another one, HS was detected with HG and 8PCHG.

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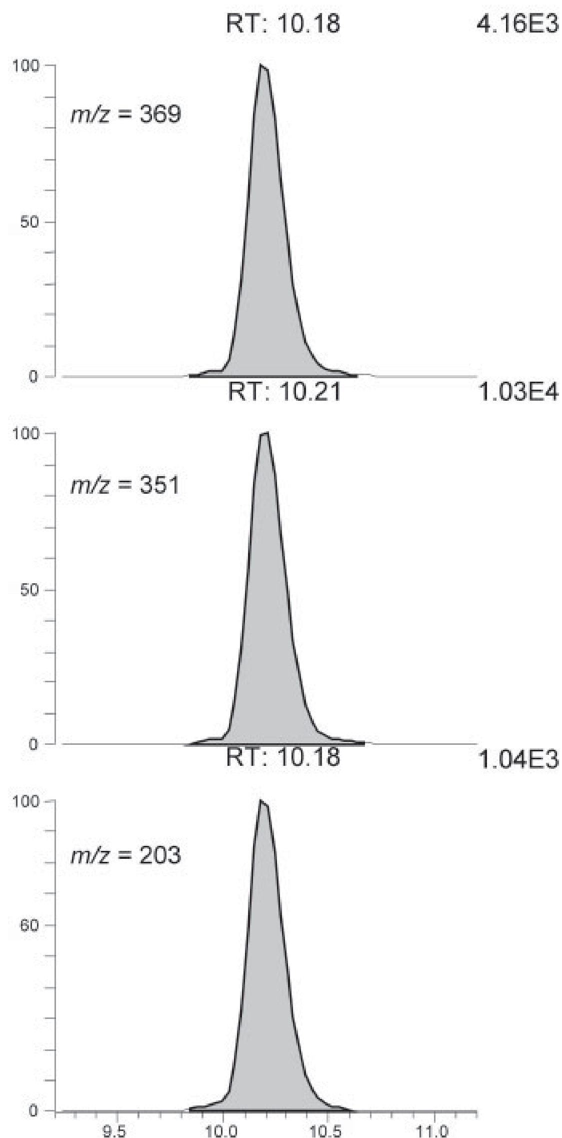

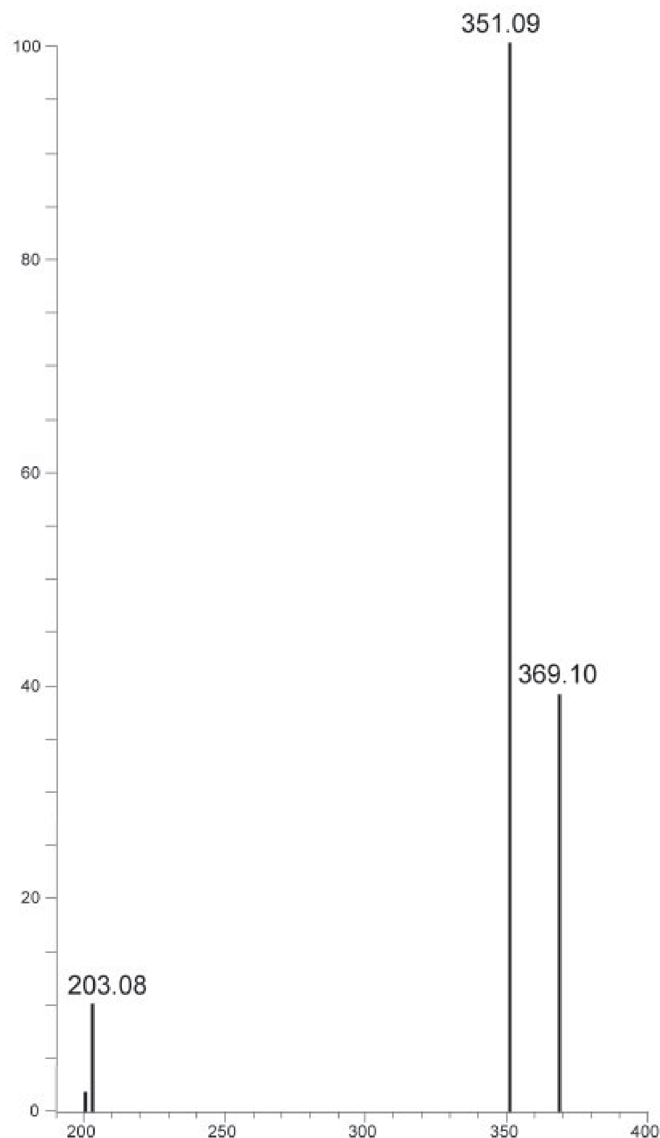

Figure 2. Extracted ion mass chromatograms (left) and spectrum (right) of harpagoside at 100 pg/μL obtained on the LTQ mass spectrometer in the full scan MS^3 mode.

Table 1. Detection of HS, HG and 8PCHG in post-competition samples (*n* = 317)

Compounds detected			Number of samples in which HS was detected
HS	8 PCHG	HG	
>1 ng/mL	Not detected	Not detected	5
>1 ng/mL	>1 ng/mL	Not detected	1
>1 ng/mL	>1 ng/mL	Detected	1
<1 ng/mL	Not detected	Not detected	1

According to our previous *Harpagophytum* study in the horse (Colas *et al.*, 2006), the presence of 8PGHG, HG and HS may be due to a recent administration of *Harpagophytum* having taken place less than 4 h before sampling.

Detection of EE in spiked urine and in post-administration urine samples

Typical ion chromatograms obtained from a blank urine sample and a urine sample spiked at 1 ng/mL are

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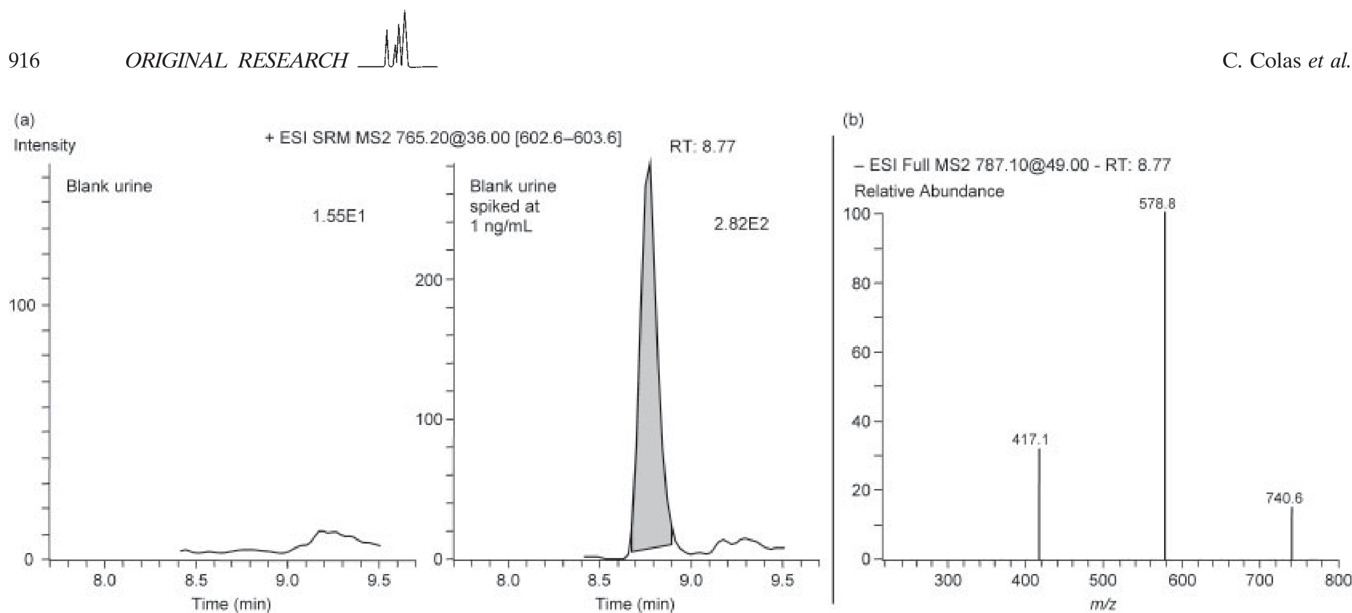

Figure 3. (a) Extracted ion chromatograms obtained in the SRM mode (on *m/z* 765) in the ESI positive mode from blank urine (left) and from spiked urine (1 ng/mL) (right). (b) Full-scan MS^2 mass spectrum of eleutheroside E from CID on *m/z* 787 in the negative ESI mode. Characteristic ions are *m/z* 741, *m/z* 579 and *m/z* 417, which correspond to formate elimination and to two successive losses of glucose, respectively.

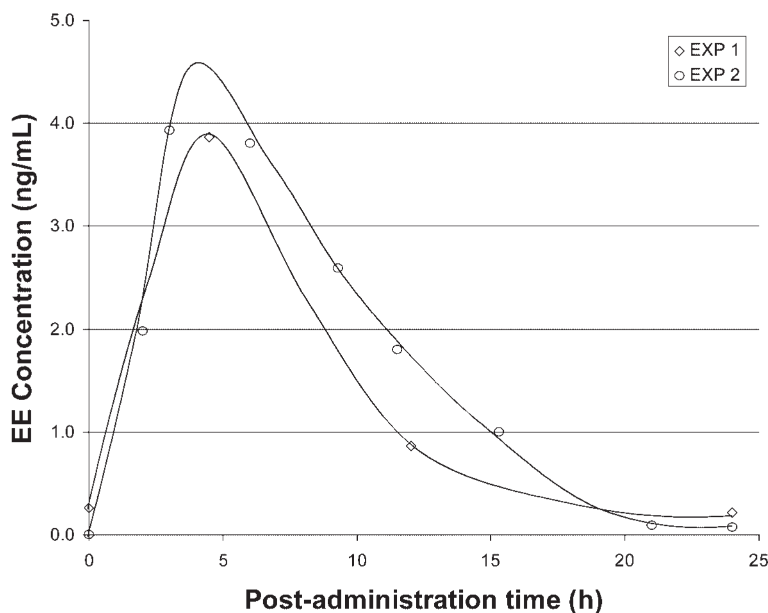

Figure 4. Elimination profile of eleutheroside E in urine after administration of two doses of Twydil® Hippacan + C for 5 days.

shown in Fig. 3. The EE extraction recovery was evaluated on eight urine samples at two concentrations: 2 and 20 ng/mL. The recovery yield was 85% at 2 ng/mL (RSD 11%) and 95% at 20 ng/mL (RSD 7%).

EE concentration in urine after administration was followed on two experiments (Fig. 4). EE concentrations were estimated with 1 ng/mL EE spiked urine. As shown on Fig. 4, urine concentrations were below 5 ng/mL. Peak concentrations were observed about 5 h after administration. EE was not detectable later than 24 h after the last administration. It is important to note that EE was detected at low concentration (i.e. less than 500 pg/mL) in the urine sample collected after the last administration; EE was also detected at low level of concentration (i.e. less than 500 pg/mL) in the sample collected before the first administration. The presence of EE in the pre-administration may indicate that its presence cannot be only attributed to the ingestion of herbal dietary supplements containing *Eleutherococcus senticosus.*

Detection of EE in post-race routine urine samples (Table 2)

A total of 382 urine samples were collected from 100 show horses, 229 racing horses and 53 unknown horses

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Table 2. Detection of EE in post competition samples (*n* = 382)

			EE detection		
			Number of horses[a]		
Horse origin	Sub-group	Total number: 382 Number in each sub-group	Not detected	Trace (<1 ng/mL)	Concentration close to 1 ng/mL or higher than 1 ng/mL
Show horses	Post-race	77	32 (42)	35 (45)	10 (13)
	Out of competition	23	20 (87)	3 (13)	0
Racing horses	Post-race Thoroughbred	74	61 (82)	9 (12)	4 (6)
	Post-race harness	133	84 (63)	44 (33)	5 (4)
	Racing horses at training	22	8 (36)	13 (59)	1 (6)
Unknown		53	28 (53)	25 (47)	0

[a] Percentages in the corresponding group or sub-group are given in parentheses.

(either show horses or racing horses). As shown in Table 2, EE was not detected in a total of 233 samples corresponding to 61% of the population. In 129 samples corresponding to 34%, EE was found at traces levels. In 20 samples (5%), EE was found at a concentration higher than 1 ng/mL or close to 1 ng/mL. The concentration of one of these samples was estimated to be about 2 ng/mL.

Considering each sub-group, it is interesting to observe that the percentage of negative samples varies between 36 and 87% depending of the group or sub-group. The percentage of negative samples is higher than 80% in two sub groups: (i) the post-race thoroughbred horses sub-group in which horse feed is generally strictly controlled, and (ii) the out of competition show horse sub-group which was composed of known horses which were not fed with herbal preparations. The number of horses in each group is quite small; however these data indicate that the presence of EE cannot be only attributed to the ingestion of feed contaminants containing *Eleutherococcus senticosus*.

CONCLUSION

The HS findings in otherwise negative post-race routine samples verify that the method developed allows detection of *Harpagophytum* administration in field urine samples. Regarding the EE survey, the presence of trace levels of EE in a large percentage of urine samples was unexpected and indicates that the origin of the occurrence of EE is not well established. Detection of *Harpagophytum* and EE in feed supplements has also been developed. In the future, these methods could be extended to other chemically related compounds.

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