Optimization of Solid-Phase Extraction for the Liquid Chromatography–Mass Spectrometry Analysis of Harpagoside, 8-*para*-Coumaroyl Harpagide, and Harpagide in Equine Plasma and Urine

Cyril Colas[1,2], Patrice Garcia[1], Marie-Agnès Popot[1,*], Yves Bonnaire[1], and **Stéphane Bouchonnet[2]**

[1]LCH—Laboratoire des Courses Hippiques, 15 rue de Paradis, 91370 Verrières le Buisson, France; and [2]DCMR – Laboratoire des Mécanismes Réactionnels, Département de Chimie, École Polytechnique, CNRS, route de Saclay, 91128 Palaiseau cedex, France

Abstract

Solid-phase extraction cartridges among those usually used for screening in horse doping analyses are tested to optimize the extraction of harpagoside (HS), harpagide (HG), and 8-*para*-coumaroyl harpagide (8PCHG) from plasma and urine. Extracts are analyzed by liquid chromatography coupled with multi-step tandem mass spectrometry. The extraction process retained for plasma applies BondElut PPL cartridges and provides extraction recoveries between 91% and 93%, with RSD values between 8 and 13% at 0.5 ng/mL. Two different procedures are needed to extract analytes from urine. HS and 8PCHG are extracted using AbsElut Nexus cartridges, with recoveries of 85% and 77%, respectively (RSD between 7% and 19%). The extraction of HG involves the use of two cartridges: BondElut PPL and BondElut C18 HF, with recovery of 75% and RSD between 14% and 19%. The applicability of the extraction methods is determined on authentic equine plasma and urine samples after harpagophytum or harpagoside administration.

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 into Europe nearly a century ago. Its secondary dried, cut, tuberous roots containing harpagophyton have been included in the French Pharmacopoeia for about fifteen years.

Herbal preparations containing Harpagophytum, along with other plants, are used for the prevention of inflammatory symptoms in competition horses and in the treatment of animals with lameness. According to Andersen et al., Harpagophytum procumbens acts by the migration of interleukins and leucocytes to the painful and inflamed joint area and shows anti-inflammatory, analgesic, and antiphlogistic properties (1). As a result of a number of recent studies in rats and men, Harpagophytum is proposed as a complementary treatment for chronic rheumatisms, tendinitis, osteoarthritis, and arthritis (1–5). Harpagophytum acts at the muscular level; therefore, sportsmen use it to avoid or ease pain due to heavy effort.

Chemically, Harpagophytum contains a number of compounds, especially iridoid glycosides like harpagoside, harpagide, and 8-para-coumaroyl harpagide, which are displayed in Figure 1 (6). Apart from recent studies such as those by Seger et al. and Baranska, there is little information in the literature on the analysis and extraction of these compounds from plants and biological fluids (7,8). The usual scheme of drug screening in plasma or urine involves several automated solid-phase extraction (SPE) methods and/or liquid–liquid extraction (LLE) that cover a wide range of drugs and metabolites (9,10). The analysis of the extracts generally requires gas chromatography (GC) or liquid

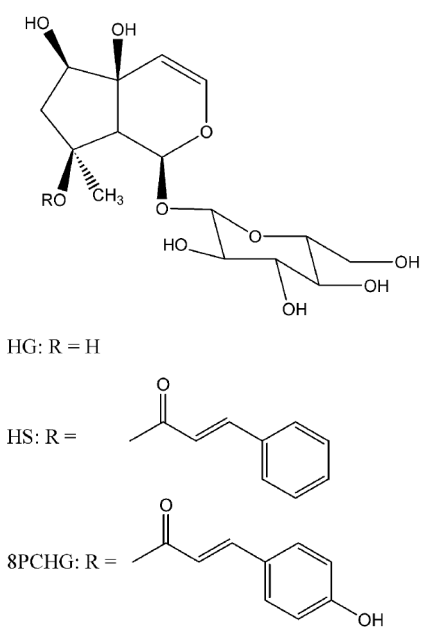

Figure 1. Chemical structures of harpagide (HG), harpagoside (HS), and 8-para-coumaroyl harpagide (8PCHG).

*Author to whom correspondence should be addressed: email: mariepopot@wanadoo.fr.

chromatography (LC) coupled with mass spectrometry (MS). Because LC–MS is known to be very sensitive to ion suppression, it is important to eliminate the maximum of interfering compounds during the extraction process.

SPE has been used for more than a decade and has evolved as a powerful technique for the extraction of a wide range of drugs from plasma and urine (11,12). It generally offers many advantages over LLE, such as higher selectivity, better reproducibility, and the possibility of using smaller sample volumes. Horse urine is a complex matrix that blocks frequently polymeric materials considered to be more resistant than silica (13). However, C18 bonded silica is still frequently used, especially for the extraction of anabolic steroids, corticosteroids, and other drugs. According to Wynne, a strong cation exchange or a mixed-mode (strong cation exchange + non polar C8) cartridge can be used for the screening of basic compounds of different polarities (14). To reduce the cost of analysis, a single SPE has also been proposed to prepare both an "acid-neutral" extract and a basic extract suitable for horse urine drug screening (15).

The aim of this study was to optimize the extraction of harpagoside, harpagide, and 8-para-coumaroyl harpagide from plasma and urine. An analytical method involving the coupling of liquid chromatography with multi-step tandem mass spectrometry was developed; it was used to compare the recoveries and selectivity of the different extraction procedures.

Experimental

Chemicals

HPLC-grade water was obtained by purifying water in a Milli-Q filtration system (Millipore, Bedford, MA). HPLC-grade methanol, *n*-hexane, acetonitrile, ethyl acetate, and ammonia solution 30% were obtained from Carlo Erba (Milan, Italy); diethyl ether and chloroform from Riedel-de Haën (Seelze, Germany); isopropyl alcohol, acetic acid 100%, hydrochloric acid 37%, phosphoric acid 84%, formic acid, and salts for buffers from VWR (Paris, France); and methylene chloride from JT Baker (Deventer, The Netherlands).

Harpagoside (HS) was supplied by Extrasynthèse (Genay, France); harpagide (HG), and 8-para-coumaroyl harpagide (8PCHG) were purchased from Phytolab (Hamburg, Germany). Hydroxylidocaine (OHL), used as the internal standard for the LC–MS quantitation, was synthesized at our laboratory (16,17). Stock solutions of standards were prepared by dissolving the selected analytes in methanol; they were stored at 4ºC before use.

Harpadol from Arkopharma (Carros, France) and Harpagophyt from CTB Equitop (Le Perray, France), commercial preparations containing Harpagophytum, were used for administration to horses.

Solid-phase extraction

SPE was performed with a Rapid Trace SPE workstation (Zymark, Hopkinton, MA) equipped with 3-mL cartridges. C18 bonded silica BondElut C18 HF (500 mg) from Varian (Palo Alto, CA) and C8 bonded silica Isolute C8 (200 mg) from IST (Glamorgan, UK) were tested as reverse phases. Polymeric sorbents, mostly polystyrene-divinylbenzene (PS-DVB) copolymers such as Isolute ENV+ (50 and 100 mg) from IST, Oasis HLB (60 mg) from Waters (Milford, CA), BondElut PPL (100 and 200 mg), and AbsElut Nexus (60 mg) from Varian were also used. Extractions were also performed using other sorbents such as covalently bond BondElut PBA (500 mg) from Varian or cation exchange mixed-mode cartridges CSDAU 503 (500 mg) from UCT (Bristol, UK), BondElut Certify (500 mg) from Varian and Oasis MCX (60 mg) from Waters.

LC–MS^n conditions

Extraction recoveries were determined by LC–MS^n on a LCQ Deca ion trap mass spectrometer coupled with a Surveyor MS pump and a Surveyor autosampler (Thermo Electron, San Jose, CA). The analytical column used was a Zorbax Eclipse XDB-C18 Solvent Saver Plus (3.5 µm, 150 × 3.0 mm) (Agilent Technologies, Massy, France). Flow rate was of 300 µL/min with a gradient of methanol and 0.1% aqueous formic acid solution (pH 2.6): 10% methanol 0–2 min, 60% at 4 min, 80% at 5 min, 100% from 10 to 15 min and 10% from 16 to 20 min. 10 µL of the sample were injected.

The mass spectrometer was operated in the electrospray ionization (ESI) positive mode; 4.5 kV were applied to the ESI needle. The flows of sheath and auxiliary gas were set at 80 and 10 arbitrary units, respectively. The heated capillary temperature was maintained at 200°C. Analytes were detected in the MSn mode, with a qz factor of 0.25 for all precursor ions. Table I gives the main parameters associated with the collision-induced dissociation (CID) step for each compound of interest. Our laboratory is subject to AORC (Association of Official Racing Chemists) guidelines, which prescribe minimum criteria for the identification of analytes (18,19). These minimum criteria imply at least 3 characteristic ions with

Table I. MS^n Parameters for Detection of HG, HS, and 8PCHG with the LCQ Deca Mass Spectrometer

	Analyte	HG	HS	8PCHG
MS^2	Parent ion (*m/z*)	387.0	517.1	533.1
	Isolation width (*m/z*)	1.0	1.0	1.8
	Collision energy (%)*	26.0	23.5	23.5
	m/z scanning range	180.0–400.0	200.0–530.0	200.0–550.0
	Main fragment ions (*m/z*) and relative abundances (%)	369.0 (40)–207.0 (100) 203.0 (60)	517.0 (40)–369.0 (100)	533.0 (70)–369.0 (100)
MS^2	Parent ions (*m/z*)		369.0	369.0
	Isolation width (*m/z*)		1.0	1.0
	Collision energy (%)*		21.0	22.0
	m/z scanning range		200.0–380.0	200.0–380.0
	Main fragment ions (*m/z*) and relative abundances (%)		369.0 (100)–351.0 (80)	369.0 (30)–351.0 (100)

* Arbitrary units.

relative abundance ≥ 10% in the full scan spectrum. If these conditions cannot be fulfilled, the AORC rules allow the use of two complementary techniques such as full scan MS^2 and full scan MS^3. Each technique must involve at least two characteristic ions. Thus, the detection of HG was performed in MS^2, while HS and 8PCHG had to be detected in MS^2 and MS^3 because MS^2 provided insufficient structural information (only one daughter ion) for both analytes (20).

The LCQ Deca MS provided detection limits low enough to quantitate the relatively large amounts of analytes resulting from the extraction of spiked samples. This instrument was suitable for the first step of this study devoted to the comparison of recoveries of different cartridges. However, our initial results showed that the analytical performances required for detecting the analytes at their observed concentrations in horse biological fluids would require a better sensitivity (20). A column with 1.8 µm particles was tested to enhance sensitivity but was not retained because it provided chromatographic peaks too narrow to be well plotted given the scan rate of the MS. A LC–MS^n LTQ linear ion trap MS (Thermo Electron) coupled with an Agilent 1100 Series binary pump, standard autosampler, vacuum degasser and a thermostated column compartment (Agilent, Palo Alto, CA) was thus used to quantitate the analytes during the validation step of the selected extraction method. The LC method was similar to the one described previously, but the flow rate was set to 400 µL/min instead of 300 µL/min. The column was equipped with a Zorbax Eclipse XDB-C18 (5 µm, 12.5 × 4.6 mm) analytical guard column and a 0.5 µm high pressure frit.

The mass spectrometer was operated in the ESI positive mode, and 4.5 kV were applied to the ESI needle. The flows of sheath and auxiliary gas were set at 40 and 5 arbitrary units, respectively. The capillary temperature was maintained at 350°C. The qz factor was set to 0.25 for all MSn experiments. Table II gives the main parameters associated with the CID step for each compound of interest. OHL, used as internal standard, was detected in selected reaction monitoring (SRM) mode using one transition. The detection of HG was performed in full scan MS2, whereas the detection of HS and 8PCHG was performed in full scan MS3. Quantitation was carried out in the consecutive reaction-monitoring (CRM) mode on two consecutive transitions. CRM is a very selective mode, similar to SRM in MS3.

Extraction procedure

Plasma

Three milliliters of plasma (from 4 to 10 sample pool) were diluted with 2.5 mL of water, and 200 ng of standards in methanol (20 µL) were added. The mixture was centrifuged for 30 min at 3000 g. The supernatant was transferred to a clean tube and 2 mL of water were added. SPE was then automatically performed following the procedures detailed in Table III.

Table II. MS^n Parameters for Detection of OHL, HG, HS, and 8PCHG with the LTQ Mass Spectrometer

	Analyte	OHL	HG	HS	8PCHG
	Mode	SRM	MS^2	CRM	CRM
MS^2	Parent ion (*m/z*)	251.1	387.1	517.1	533.1
	Isolation width (*m/z*)	1.0	1.4	1.7	1.7
	Collision energy (%)*	30.0	25.0	25.0	25.0
MS^3	Parent ion (*m/z*)			369.1	369.1
	Isolation width (*m/z*)			1.3	1.3
	Collision energy (%)*			25.0	25.0
	m/z scanning range	85.5–86.5	180.0–400.0	350.6–351.6	350.6–351.6
	Main fragment ions (*m/z*) and relative abundances (%)	86.0	269.0 (40)–207.0 (100) 203.0 (60)	351.1	351.1

* Arbitrary units.

Table III. Procedures to Perform SPE on Plasma Samples

	Cartridge	Conditioning		Washing / Drying		Elution		Ref.
(a)	Oasis HLB	MeOH	2 mL	H_2O	3 mL	MeOH/EtOAc (50/50)	3 mL	
		H_2O	2 mL	MeOH/H_2O (30/70)	3 mL			
				drying	3 min			
				n-hexane	3 mL			
				drying	3 min			
(b)	Isolute ENV+	MeOH	3 mL	H_2O	3 mL	MeOH/CH_3CN (50/50)	3 mL	(21)
		H_2O	3 mL	drying	5 min			
(c)	AbsElut Nexus	MeOH	2 mL	H_2O	3 mL	CH_2Cl_2/iPrOH (80/20)	3 mL	(22,23)
		H_2O	2 mL	MeOH/H_2O (30/70)	3 mL			
				drying	5 min			
(d)	Focus	*Cf.*(b) Isolute ENV+						
(e)	BondElut PPL	MeOH	6 mL	H_2O	3 mL	MeOH/CH_3CN (50/50)	3 mL	(23–25)
		H_2O	12 mL	drying	8 min			

Urine

The tests on urine extraction were performed with a mixture of 6 to 12 individual urine collections in order to obtain a representative matrix. As a matter of fact, the great variability of the matrix composition, especially in the case of urine, can significantly influence recovery ratios. The composition of equine urine depends of the horse being considered. Indeed, it is strongly correlated to several factors such as feed, time of collection, season, exercise, and pH which may vary between 4.5 and 10 (26).

Five milliliter urine samples were spiked with 200 µL of a standard solution at 10 ng/µL (i.e., 2 µg), and 2 mL of appropriate buffer were added (the pH was adjusted according to the cartridge tested). The sample was centrifuged at 3000 g for 30 min before SPE, in accordance with the procedures detailed in Table IV.

Solid-phase extraction

SPE was performed with a Rapid Trace workstation. Drying was done with a nitrogen flow. Conditioning was performed at 6

Table IV. Procedures to Perform SPE on Urine Samples

	Cartridge	pH	Conditioning		Washing / Drying		Elution		Ref.
(f)	BondElut C18 HF	9.2	MeOH H_2O	5 mL 5 mL	H_2O drying n-hexane drying	5 mL 3 min 3 mL 3 min	CH_2Cl_2/iPrOH 80/20	3 mL	(23)
(g)	Isolute C8	7.1	MeOH H_2O	5 mL 5 mL	H_2O drying n-hexane drying	3 mL 3 min 3 mL 3 min	$CHCl_3$	3 mL	
(h)	CSDAU 503	6.1	MeOH PO_4 buffer - pH 6.1	2 mL 2 mL	CH_3CO_2H 1M drying drying	1 mL 5 min 3 min	EtOAc/NH_4OH 98/2 MeOH	2 mL 3 mL	(27–29)
(i)	BondElut Certify		Cf. CSDAU 503						
(j)	Oasis MCX	6.1	MeOH H_2O	2 mL 2 mL	HCl 0.1M drying MeOH drying	2 mL 5 min 2 mL 3 min	CH_3CN/iPrOH/ NH_4OH 40/60/4	2 mL	
(k)	BondElut PBA*	8.6	H_3PO_4 2M in MeOH H_2O $SO_3(NH_4)_2$ buffer - pH 8.4	6 mL 6 mL 6 mL	$SO_3(NH_4)_2$ buffer - pH 8.4	3 mL	MeOH/H_2O/ CH_3CO_2H 90/5/5	8 x 3 mL	
(l)	Isolute ENV+	7.1	MeOH H_2O	2 mL 2 mL	MeOH/H_2O 5/95 drying	3 mL 3 min	MeOH/H_2O 80/20	2 x 2.5 mL	(21)
(m)	Oasis HLB	8.0	MeOH H_2O	2 mL 2 mL	H_2O MeOH/H_2O 30/70 drying *n*-hexane drying	3 mL 3 mL 3 min 3 mL 3 min	CH_2Cl_2/iPrOH 80/20	3 mL	
(n)	BondElut PPL	7.4	MeOH H_2O	6 mL 12 mL	H_2O drying	3 mL 8 min	MeOH/CH_3CN 50/50	3 mL	(23-25)
(o)	AbsElut Nexus	7.5	Cf. (m) Oasis HLB						(22,23)
(p)	BondElut C18 HF + BondElut PPL	7.5	MeOH H_2O The pool of the loading and washing fractions was then extracted on the second cartridge Cf. (n) BondElut PPL	5 mL 5 mL	H_2O	1 mL			
(q)	BondElut PPL + BondElut C18 HF	7.5	Cf. (n) BondElut PPL After evaporation, the residue was dissolved in 100 µL of MeOH to which 3 mL of H_2O had been added MeOH H_2O	6 mL 6 mL	drying *n*-hexane drying MeOH/H_2O 30/70	5 min 4 mL 3 min 3 mL	MeOH/H_2O 50/50	3 mL	

* Loading step at 0.3 mL/min (0.8 mL/min in all other cases).

mL/min, loading at 0.8 mL/min, rinsing at 3 mL/min, and collecting at 0.8 mL/min. Solvents used for these steps are detailed in Tables III and IV. After extraction, samples were evaporated to dryness at 48°C with a TurboVap LV Evaporator (Zymark). The residual extract was reconstituted in 200 µL of a 50/50/0.1 MeOH/H_2O/HCO_2H mixture and analyzed by LC–MS^n.

Extraction recoveries

Apparent extraction recoveries

In what follows, the term "apparent extraction recovery" is used for an extraction recovery estimated by comparing the chromatographic signal obtained for the plasma or urine extract to the one obtained for the standard solution in the same solvent. This "apparent extraction recovery" takes into account the "real extraction recovery", which does not include the matrix effect affecting LC–MS analysis; it provides information on the selectivity of the entire method.

Real extraction recoveries

Accurate determination of real extraction recoveries would require performing quantitation with internal standards corresponding to labeled analytes, but such standards are not available. Real extraction recoveries of HG, HS, and 8PCHG were determined using OHL as the internal standard. This measurement was performed on twelve different biological samples according to the following procedure. Three 1 mL aliquots of non-spiked samples, one 1 mL aliquot spiked with x ng of standards, and one 1 mL aliquot spiked with 10x ng were extracted on the selected SPE cartridges (x is equal to 0.5 for plasma and 2 for urine). After extraction, x ng of standards were added to one of the non-spiked samples; 10× ng to another one. For HG in urine, 2 additional samples fortified after the first extraction were used in order to determine the real extraction recoveries for each SPE cartridge. 2.5 ng of internal standard were added to each one of the extracts. The extracts were then evaporated to dryness and reconstituted in 25 µL of methanol and 175 µL of acidified water (0.1% of formic acid) before analysis by LC–MSn on the LTQ mass spectrometer. The absence of analyte chromatographic peaks was checked in the blank sample.

Table V. Apparent Extraction Recoveries in Plasma Using Different SPE Cartridges

Cartridge	Extraction method*	Apparent extraction recoveries† (%)			Comments
		HG	HS	8PCHG	
Oasis HLB	(a)	nd‡	83	60	The apparent extraction recoveries obtained for HS and 8PCHG are acceptable but HG is not sufficiently retained on the cartridge.
Isolute ENV ± 50 mg	(b_1)	54	64	72	The sorbent (hydroxylated PS-DVB copolymer) allows the extraction of the three molecules in plasma.
Isolute ENV ± 100 mg	(b_2)	55	67	68	
AbsElut Nexus	(c)	nd	56	39	HG is not retain on the sorbent (modified PS-DVB copolymer) and is eluted as soon as the sample is loaded on the column.
Focus	(d)	nd	81	69	The apparent extraction recoveries obtained for HS and 8PCHG are acceptable but HG is not retained on this sorbent (modified PS-DVB copolymer).
BondElut PPL–100 mg	(e_1)	86	85	69	The sorbent (modified PS-DVB copolymer) allows the extraction of the three molecules in plasma with great apparent extraction recoveries.
BondElut PPL–200 mg	(e_2)	100	102	96	

* Extraction methods are detailed in Table III.
† Apparent extraction recoveries were calculated on at least 3 plasma samples.
‡ nd: not detected.

Results and Discussion

For each cartridge, different elution solvents among those currently used for SPE were tested. In order to optimize extraction procedures (i.e., to get an acceptable recovery), all the solvent fractions were collected for the determination of appropriate volumes. Only the best results are presented in the following.

Plasma

Comparison of SPE cartridges

In order to select an appropriate cartridge for the extraction of these compounds in plasma, different sorbents were tested for plasma extraction, and apparent extraction recoveries are given in Table V. Oasis HLB (cartridges routinely used in our laboratory) were first tested, then Isolute ENV+, a sorbent developed for environmental purposes and able to retain very polar analytes. Other modified PS-DVB copolymers were also tested (i.e., AbsElut Nexus, Focus, and BondElut PPL), and BondElut PPL was selected.

Further optimization on the plasma extraction procedure with PPL BondElut cartridges

The results previously mentioned indicate that both Isolute ENV+ and BondElut PPL cartridges allow the simultaneous extraction

of the three analytes. The BondElut PPL (200 mg) was selected for further optimization because it provided the best apparent extraction recoveries (25% greater for 8PCHG and 15% greater for HG and HS than the 100 mg BondElut PPL cartridge).

Table VI. Real Extraction Recoveries of Analytes from Plasma Using BondElut PPL Cartridges (Mean Values for Extracts from 12 Different Samples)

	HG		HS		8PCHG	
Spiked concentrations (ng/mL)	0.5	5	0.5	5	0.5	5
Recoveries (%)	92	92	93	88	91	94
RSD (%)	8	8	8	7	13	7

Influence of the volume of plasma

Different volumes of plasma (at 2 ng/mL in each analyte) from 1 to 5 mL were submitted to the extraction procedure. Figure 2 shows that, for HG and HS, the extracted quantities are proportionally correlated to the plasma volume loaded on the cartridge in the considered range. For 8PCHG, saturation of the sorbent is observed for volumes greater than

Table VII. Apparent Extraction Recoveries in Urine Using Different SPE Cartridges

Sorbent type	Cartridge	Extraction method*	Apparent extraction recoveries† (%)			Comments
			HG	HS	8PCHG	
Reverse Phase	BondElut C18 HF	(f)	nd	47	21	HG is not detected and the apparent extraction recoveries obtained for HS and 8PCHG are insufficient.
	Isolute C8	(g)	nd	52	25	
Mixed-Mode	CSDAU 503	(h)	nd	nd	–	The very poor apparent extraction recoveries show that the considered analytes are not ionisable and only the hydrophobic interactions are involved in the retention process. There is no result for 8PCHG since this analyte was not available when these tests were performed.
	BondElut Certify	(i)	nd	5	–	
	Oasis MCX	(j)	nd	15	–	
Covalent bonded	BondElut PBA	(k)				Unlike with the other sorbent tested, the problem concerns analyte releasing: elution required too large amounts of solvent (29).
Non-polar copolymer	Isolute ENV+	(l)	nd	40	32	Comparison with apparent extraction recoveries obtained for plasma on Isolute ENV+ and BondElut PPL suggests that ionic suppression may be responsible of the non detection of HG in urine. Oasis HLB and AbsElut Nexus cartridges provide correct apparent extraction recoveries for HS and 8PCHG.
	Oasis HLB	(m)	nd	63	47	
	BondElut PPL	(n)	nd	22	21	
	AbsElut Nexus	(o)	nd	68	45	
Association of cartridges	C18 HF + PPL	(p)		–	–	The apparent extraction recovery of a urine fortified with HG before the BondElut PPL extraction is 90%, then the BondElut C18 HF cartridge is able to retain interferences responsible for ionic suppression.
	PPL + C18 HF	(q)	24	–	–	The elution fraction from the BondElut PPL contains less matrix interferents than urine and can be well extracted on a BondElut C18 HF cartridge.

* Apparent extraction recoveries were calculated on at least 3 urine samples.
† Extraction methods are detailed in Table IV.

2.5 mL. A 1 mL sample volume was retained for routine analysis, but these tests showed that the sample volume could be increased to 2.5 mL in case of a lack of sensitivity of the LC–MS system.

pH adjustment

In most extraction procedures, plasma samples are acidified with phosphoric acid (100 µL H_3PO_4 84% diluted 1/5 for 3 mL of plasma) to denature binding proteins (30). In our study, tests were carried out on post-administration samples; they showed that apparent extraction recoveries are not significantly modified (less than 10%) according to the sample pH, which was either pH 3 after addition of phosphoric acid or pH 7.5 in normal plasma. Consequently, the acidification step was removed from the extraction procedure of HG, HS, and 8PCHG.

Selectivity and limits

As shown in Figure 3, no interfering peak is observed around the expected retention time of HS. Results are fully comparable for HG and 8PCHG.

The linearity range was defined up to 200 ng/mL. The limit of detection (LOD) and the limit of quantitation (LOQ) measured on the LCQ Deca ion trap MS were less than 0.2 ng/mL in plasma.

Real extraction recoveries of the selected method

Table VI gives the real extraction recoveries and RSD (relative standard deviation) for the three analytes in the spiked plasmas. The results are satisfying, because recoveries are between 91% and 93% at 0.5 ng/mL and between 88% and 94% at 5.0 ng/mL, with RSD values between 8% and 13% at 0.5 ng/mL and 7 to 8% at 5.0 ng/mL.

Urine

Comparison of SPE cartridges

Several cartridges tested for plasma (i.e., isolute ENV+, Oasis HLB, BondElut PPL, and AbsElut Nexus) were also tested for urine. Additionally, BondElut C18 HF (routinely used in our laboratory) Isolute C8, several mixed-mode cartridges such as BondElut Certify or Oasis MCX, and covalent bonded sorbent BondElut PBA were also performed.

These different cartridges tested for urine extraction are given in Table VII with the apparent extraction recoveries determined for each analyte. Results are commented in Table VII for each kind of cartridge.

Final optimization of the extraction procedure with AbsElut Nexus cartridges

AbsElut Nexus and Oasis HLB cartridges allow good apparent extraction recoveries for HS (65%) and 8PCHG (45%) from urine. Although both cartridges provided similar recovery ratios, the AbsElut Nexus was chosen because it led to cleaner extracts. The specific surface area of AbsElut Nexus (650 m2/g), inferior to that of the Oasis HLB (830 m2/g), can explain why fewer matrix interferents are retained on the sorbent during the washing steps and finally desorbed during the elution step.

In order to improve the extraction efficiency, the influence of the sample volume and the pH adjustment was studied.

Volume adjustment

Different sample volumes (at a concentration of 10 ng/mL in analytes) in the range from 250 µL to 5 mL were extracted following the procedure previously described, with a constant loading volume of 10 mL. Figure 4 plots the chromatographic

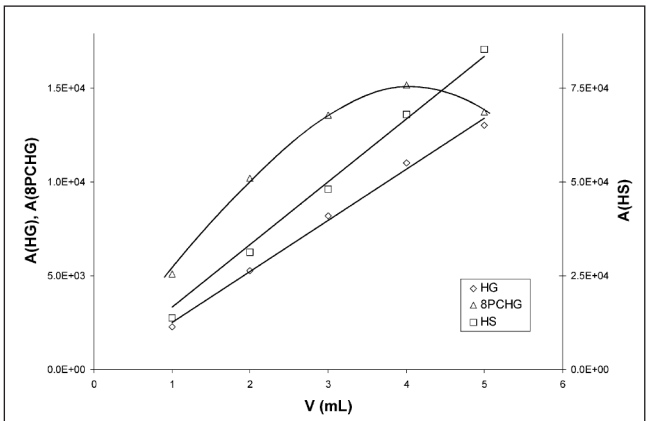

Figure 2. Influence of plasma volume on chromatographic peak area (measured with the LTQ mass spectrometer). All samples were spiked at 2 ng/mL in each analyte.

Table VIII. Real Extraction Recoveries of Analytes from Urine (Mean Values for Extracts from 12 Different Samples)

	HG		HS		8PCHG	
Extraction cartridge	PPL + C18		Nexus		Nexus	
Spiked concentrations (ng/mL)	2	20	2	20	2	20
Recoveries (%)	75	74	86	84	78	75
RSD (%)	19	14	7	12	8	19

Table IX. Detection of HG, HS, and 8PCHG in Equine Plasma and Urine after Administration of Harpagoside or Related Commercial Products

	Plasma*			Urine†		
	HG	HS	8PCHG	HG	HS	8PCHG
EXP 1‡	–	+	–	–	+	+
EXP 2‡	+	+	+	–	+	+
EXP 3‡	+	+	+	–	+	+
EXP 4‡	–	+	–	–	+	–
EXP 5‡	–	+	–	–	+	–

* 2 Hours after administration.
† 10 Hours after administration.
‡ EXP 1: 2 doses of Harpagophyt; EXP 2: 16 capsules of Harpadol; EXP 3: 32 capsules of Harpadol; EXP 4: 120 mg of harpagoside; EXP 5: 600 mg of harpagoside.

peak areas of HS and 8PCHG, reported to that of the internal standard, OHL, as a function of the urine volume. In both cases, the decrease of the chromatographic response may be attributed to the ion suppression effect during electrospray ionization. This illustrates the strong importance of the cleanness of extracts for the compounds of interest. As a good compromise for both analytes, a convenient sample volume of 1 mL was retained for further analysis.

pH adjustment

The adjustment of pH is determinant in the cleaning of the extracts (Figure 5). Extractions were achieved on 1 mL of blank urine. The samples were adjusted to different pH in the range from 4.5 to 9.5. They were spiked with 10 ng of HS and 8PCHG after extraction, so that the amounts of analytes are the same in all samples; the variations observed are thus only due to ion suppression. At pH below 6.5, the matrix effect is very strong for both HS and 8PCHG.

The same experiment was conducted with spiked urine at 10 ng/mL and the results obtained are comparable to those previously described. The real extraction recoveries were estimated; they are constant on the pH range from 4.5 to 9.5 (70 and 65% for HS and 8PCHG, respectively). A pH between 7.5 and 9 allows reproducible results to be obtained without strong ion suppression. In the developed method, the pH was set to 7.7 ± 0.1, because it is an easy value to reach in urine with a phosphate buffer.

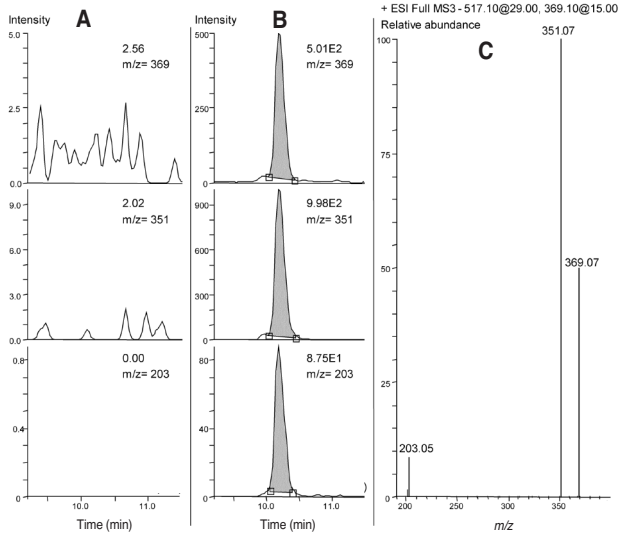

Figure 3. Selectivity of harpagoside in plasma. Extracted ion mass chromatograms at *m/z* 369, *m/z* 351, and *m/z* 203, corresponding to the harpagoside fragmentations in the MS3 mode on the LTQ mass spectrometer, obtained from (A) 1 mL of blank plasma, and (B) 1 mL of plasma spiked at 2 ng/mL. (C) Mass spectrum of harpagoside.

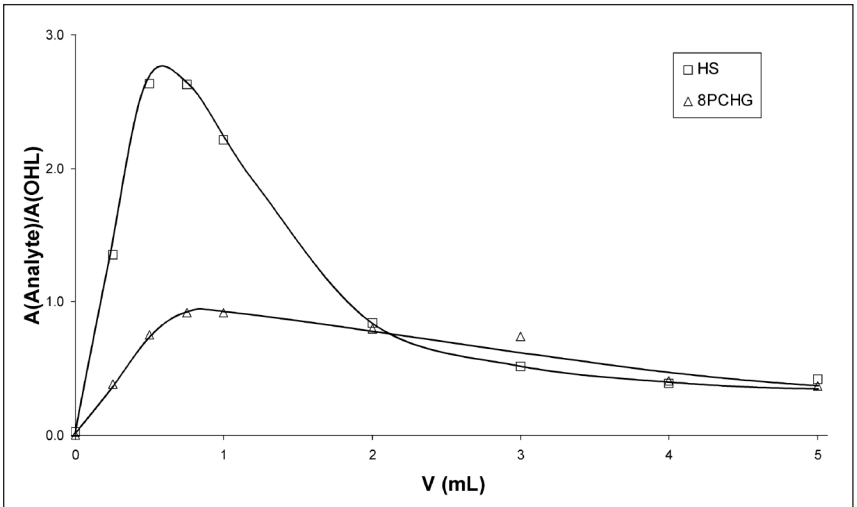

Figure 4. Influence of urine volume on chromatographic peak area (measured with the LTQ mass spectrometer). All samples were spiked at 10 ng/mL in each analyte.

Selectivity and limits

Figure 6 compares the chromatographic profiles (MS3 detection) obtained from a blank urine extract and from an extract of urine spiked with HS at 10 ng/mL before extraction. As shown in Figure 6, no interfering peak was observed in the expected retention time window of HS. Results are comparable for HG and 8PCHG (not shown).

The linearity range was defined up to 200 ng/mL. The mean LOD and LOQ of this method were estimated at about 0.2 and 0.5 ng/mL in urine, respectively, but these values are matrix-dependent.

Real extraction recoveries of the selected methods

After having selected and optimized the extraction methods (o) and (q), the real extraction recoveries were determined for HG, HS, and 8PCHG.

Table VIII shows the real extraction recoveries for HG for the complete method. Extraction recoveries are acceptable: 75% at 2 and 20 ng/mL, with RSD values between 14 and 19%. RSD values are slightly high but acceptable considering the complexity of the matrix. Real extraction recoveries were determined for each cartridge, giving 90% for the BondElut PPL (in accordance with recoveries observed for plasma) and 83% for the BondElut C18 HF. A loss of 7% is imputable to the rinsing step with BondElut C18 HF. It is to be noted that mixing both fractions before analysis induced an ion suppression effect so strong that HG became difficult to detect Table IV.

The real extraction recoveries of HS and 8PCHG are reported in Table VIII. There is no significant difference between samples at 2 ng/mL and samples at 20 ng/mL. Extraction recoveries are satisfying: 85 and 77% for HS and 8PCHG, respectively, with RSD values between 7 and 19%.

Application of the methods to post-administration samples

The methods previously selected for plasma and urine were applied on each medium collected from horses dosed with harpagoside or related commercial products, further to five different experiments. The three compounds

were identified in post-administration samples (Table IX). HS was detected in all the 2 hour post-administration plasma samples and in the 10 hour post-administration urine samples. HG and 8PCHG were detected in some urine samples and HG was detected in two plasma samples. The identification criteria were those described in Table II and Figures 3 and 6. Additionally, HS was quantitated using the method of standard additions, and further details are given elsewhere (20).

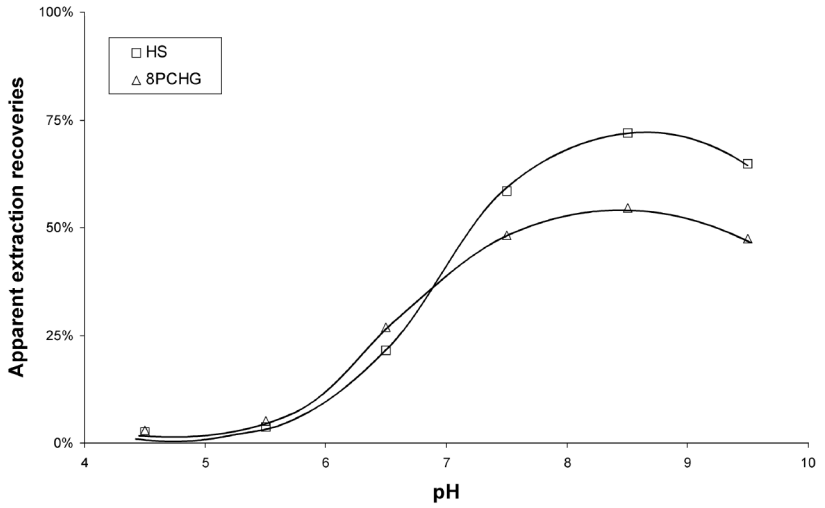

Figure 5. Influence of urine pH on apparent recoveries—ion suppression effect with the electrospray source. All samples were spiked after extraction with 10 ng of harpagoside and 8-*para*-coumaroyl harpagide.

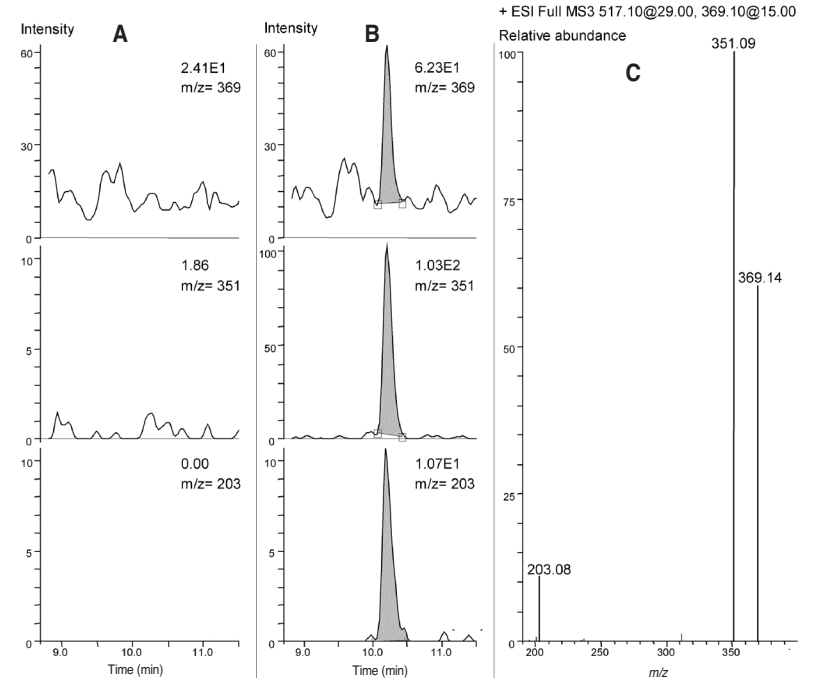

Figure 6. Selectivity of the detection of harpagoside in urine. Extracted ion mass chromatograms at m/z 369, *m/z* 351, and *m/z* 203, corresponding to the harpagoside fragmentations in the MS3 mode on the LTQ mass spectrometer, obtained from (A) 1 mL of blank urine, and (B) 1 mL of urine spiked at 10 ng/mL. (C) Mass spectrum of harpagoside.

Conclusion

The combination of SPE with LC–ESI-MSn provided an efficient and sensitive method for the detection of harpagoside, 8-*para*-coumaroyl harpagide, and harpagide in equine plasma and urine. This is the first report on an extraction method for the detection of these compounds in biological fluids by LC–MS. Among all the cartridges tested, the BondElut PPL was selected for the detection of harpagoside, 8-*para*-coumaroyl harpagide and harpagide in plasma, the AbsElut Nexus cartridge for the detection of harpagoside and 8-para-coumaroyl harpagide in urine, and the combination of BondElut PPL and BondElut C18 HF was proposed for the detection of harpagide in urine. These methods requires 1 mL of plasma or urine. Each extraction can be fully automated and requires approximately 45 min, which makes possible the analysis of a sample set within one day. This is applicable on a large scale. Further experiments have shown that quantitation of HS is possible using the method of standard additions.

Thanks to the recent development of solid-phase sorbents, several extraction methods tested here could be applicable for the detection of other polar related compounds in biological fluids.

Acknowledgments

This work was funded by ANRT and by "Les Haras Nationaux", the French Ministry of Agriculture, Food Trade, Fishing, and Rural Business. The authors are indebted to Dr. Garin and the staff for assistance with the animal experiments.

References

1. M.L. Andersen, E.H. Santos, L. Seabra Mde, A.A. Da Silva, and S. Tulfik. Evaluation of acute and chronic treatments with Harpagophytum procumbens on Freund's adjuvent-induced arthritis in rats. *J. Ethnopharmacol.* **91(2–3):** 325–30 (2004).
2. S. Chrubasik. Osteoarthritis: pharmacology and clinical indications of selected botanicals. *The Pain Clinic* **16(1):** 1–16 (2004).
3. P. Chantre, A. Cappelaere, D. Leblan, D. Guedon, J. Vandermander, and B. Fournie. Efficacy and tolerance of Harpagophytum procumbens versus diacerhein in treatment of osteoarthritis. *Phytomedicine* **7(3):** 177–83 (2000).
4. J.J. Gagnier, S. Chrubasik, and E. Manheimer.

Harpagophytum procumbens for ostheoarthitis and low back pain: a systematic review. *BMC Complement. Altern. Med.* **4:** 13 (2004).
5. I.M. Mahomed and J.A. Ojewole. Analgesic, antiinflammatory and antidiabetic properties of Harpagophytum procumbens DC (Pedaliaceae) secondary roots aqueous extract. *Phytother. Res.* **18(12):** 982–9 (2004).
6. T. Kikuchi, S. Matsuda, Y. Kubo, and T. Namba. New iridoid glycosides from Harpagophytum procumbens DC. *Chem. Pharm. Bull.* **31(7):** 2296–301 (1983).
7. C. Seger, M. Godejohann, and L.H. Tseng. LC-DAD-MS–SPE-NMR hyphenation—A tool for the analysis of pharmaceutically used plant extracts: identification of isobaric iridoid glycoside regioisomers from Harpagophytum procumbens. *Anal. Chem.* **77(3):** 878–85 (2005).
8. M. Baranska, H. Schulz, R. Siuda, M.A. Strehle, P. Rosch, J. Popp, E. Joubert, and M. Manley. Quality control of Harpagophytum procumbens and its related phytopharmaceutical products by means of NIR-FT-Raman spectroscopy. *Biopolymers* **77(1):** 1–8 (2005).
9. H.H. Maurer. Liquid chromatography-mass spectrometry in forensic and clinical toxicology. *J. Chromatogr. B* **713(1):** 3–25 (1998).
10. S. Paterson, R. Cordero, S. Mc Culloch, and P. Houldsworth. Analysis of urine for drugs of abuse using mixed-mode solid-phase extraction and gas chromatography-mass spectrometry. *Ann. Clin. Biochem.* **37(5):** 690–700 (2000).
11. P.M. Wynne, D.C. Batty, J.H. Vine, and N.J.K. Simpson. A multi-eluate approach to solid-phase extraction of biological fluids for detection of drug residues. Proceedings of the 13th International Conference of Racing Analysts and Veterinarians. R.B. Rogers, E. Houghton, and J.F. Wade, Eds. R & W Publications, Newmarket, UK, 2001, pp. 232–40.
12. P.M. Wynne, D.C. Batty, J.H. Vine, and N.J.K. Simpson. An improved method for the automated extraction of anabolic steroids from equine urine. Proceedings of the 13th International Conference of Racing Analysts and Veterinarians. R.B. Rogers, E. Houghton, and J.F. Wade, Eds. R & W Publications, Newmarket, UK, 2001, pp. 245–51.
13. P.M. Wynne, D.C. Batty, J.H. Vine, and N.J.K. Simpson. Reduced blocking rates through application of a new NC-SPE sorbent to the extraction of equine urine. Proceedings of the 13th International Conference of Racing Analysts and Veterinarians. R.B. Rogers, E. Houghton, and J.F. Wade, Eds. R & W Publications, Newmarket, UK, 2001, pp. 445–52.
14. P.M. Wynne. The application of SPE to veterinary drug abuse in solid-phase extraction. *Solid-Phase Extraction—Principles, Techniques and Applications.* N.J.K. Simpson, Eds. Marcel Dekker, New York, NY, 2000, pp. 273–306.
15. D.C. Batty, P.M. Wynne, and J.H. Vine. Comprehensive drug screening using a single solid-phase extraction cartridge. Proceedings of the 11th International Conference of Racing Analysts and Veterinarians. D.E. Auer, and E Houghton, Eds. R & W Publications, Newmarket, UK, 1996, pp. 197–202.
16. J. Thomas and P. Meffin. Aromatic hydroxylation of lidocaine and mepivacaine in rats and humans. *J. Med. Chem.* **15(10):** 1046–9 (1972).
17. J.D. Harkins, G.D. Mundy, W.E. Woods, A. Lehner, W. Karpiesiuk, W.A. Rees, L. Dirikolu, S. Bass, W.G. Carter, J. Boyles, and T. Tobin. Lidocaine in the horse: its pharmacological effects and their relationship to analytical findings. *J. Vet. Pharmacol. Ther.* **21:** 462–76 (1998).
18. Association of Official Racing Chemists, 2002, AORC guidelines for the minimum criteria for identification by chromatography and mass spectrometry, MS Criteria Working group, Internal communication to AORC members.
19. P. Van Eenoo and F.T. Delbeke. Criteria in chromatography and mass spectrometry—A comparison between regulations in the field of residue and doping analysis. *Chromatographia Suppl.* **59(S1):** S39–S44 (2004).
20. C. Colas, P. Garcia, M.A. Popot, Y. Bonnaire, and S. Bouchonnet. Liquid chromatography/electrospray ionization mass spectrometric characterization of Harpagophytum in equine urine and plasma. *Rapid. Comun. Mass Spectrom.* **20(22):** 3257–66 (2006).
21. A. Lindström, A.C. Albertsson, and M. Hakkarainen. Development of a solid-phase extraction method for simultaneous extraction of adipic acid, succinic acid and 1,4-butanediol formed during hydrolysis of poly(butylene adipate) and poly(butylene succinate). *J. Chromatogr. A* **1022(1–2):** 171–7 (2004).
22. R. Kay, S. Hines, K. Woodward, S. Hudson, and P. Teale. The use of Nexus polymeric phase extraction cartridges as a pre-extraction medium for screening and confirmatory methodologies. Proceedings of the 14th International Conference of Racing Analysts and Veterinarians. D.W. Hill, and W.T. Hill, Eds. R & W Publications, Orlando, Florida, USA, 2003, pp. 243–7.
23. M.C. Hennion. Solid-phase extraction: method development, sorbents, and coupling with liquid chromatography. *J. Chromatogr. A* **856(1–2):** 3–54 (1999).
24. P. Bartels, E. Ebeling, B. Krämer, H. Kruse, N. Osius, K. Vowinkel, O. Wassermann, J. Witten, and C. Zorn. Determination of chlorophenols in urine of children and suggestion of reference values. *Fresenius' J. Anal. Chem.* **365(5):** 458–64 (1999).
25. M.C. Hennion, C. Cau-Dit-Coumes, and V. Pichon. Trace analysis of polar organic pollutants in aqueous samples tools for the rapid prediction and optimisation of the solid-phase extraction parameters. *J. Chromatogr. A* **823(1–2):** 147–61 (1998).
26. T. Tobin. *Drugs and the Performance Horse.* Charles C. Thomas Publ. Springfield, IL, 1981.
27. M. Gravel, K.A. Thompson, M. Zak, C. Bérubé, and D.G. Hall. Universal solid-phase approach for the immobilization, derivatization, and resin-to-resin transfer reactions of boronic acids. *J. Org. Chem.* **67(1):** 3–15 (2002).
28. J. Hartleb, Y. Damm, R. Arndt, E. Christophers, and E. Stockfleth. Determination of 5-S-cysteinyldopa in plasma and urine using a fully automated solid-phase extraction-high-performance liquid chromatographic method for an improvment of specificity and sensitivity of this prognostic marker of malignant melanoma. *J. Chromatogr. B* **727(1):** 31–42 (1999).
29. D.C. Batty, N.J.K. Simpson, J.H. Vine, and P.M. Wynne. A multi-eluate approach to SPE of biological fluids for drugs of abuse analysis—Part 3. Proceedings of the 14th International Conference of Racing Analysts and Veterinarians. D.W. Hill, and W.T. Hill, Eds. R & W Publications, Orlando, Florida, USA, 2003, pp. 206–214.
30. B. Law and S. Weir. Fundamental studies in reversed-phase liquid-solid extraction of basic drugs; III: sample matrix effects. *J. Pharm. Biomed. Anal.* **10(7):** 487–93 (1992).

Manuscript received August 8, 2007;
revision received January 5, 2007.