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Automated SPE-RP-HPLC fractionation of biofluids combined to off-line NMR spectroscopy for biomarker identification in metabonomics☆

Serge Rezzi[a,*], Francia Arce Vera[a], François-Pierre J. Martin[a], Serena Wang[a], Dennis Lawler[b], Sunil Kochhar[a]

[a] BioAnalytical Science, Nestlé Research Center, PO Box 44, CH-1000 Lausanne 26, Switzerland
[b] Pet Basic Research, Nestlé Research Center-St. Louis, Checkerboard Square, St. Louis, MO 63164, USA

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ABSTRACT

NMR-based metabonomics is a valuable and straightforward approach to measuring hundreds of metabolites in complex biofluids. However, metabolite identification is sometimes limited by overlapped signals in NMR spectra. We describe a new methodology using an automated hyphenation of solid phase extraction (SPE) with RP-HPLC combined to NMR spectroscopy, which allowed identification of 72 metabolites of various molecular classes in human urine. This methodology was also successfully applied to the fractionation of a cat urine sample to aid identification of aromatic compounds and felinine. The SPE-RP-HPLC method appears to be a reliable tool to support biomarker discovery in metabonomic studies.

1. Introduction

Nowadays, modern nutrition has expanded its domain to promoting health and well-being, which requires a deeper understanding of molecular interactions between the complex food and biological processes. The explosion of nutrigenomic sciences has opened new avenues for characterizing food effects at the genome, proteome and metabolome levels. Metabonomics attempts to characterize physiological regulatory processes in living organisms by the analysis of a wide range of metabolites using nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) in biological matrices such as biofluids and tissues [1,2]. The application of statistical techniques reveals relevant metabolic regulations associated with a specific nutritional, environmental, pharmaceutical or toxicological stimulus [3,4]. Metabonomic outcomes often appear as a series of concentration changes that are indicative of modulations of specific pathways from the host and its symbiotic microbial partners [2,5–7]. A cornerstone in metabonomics is therefore the unambiguous identification of the metabolites. NMR spectroscopy is a technique of choice to achieve this since many metabolites can be simultaneously screened with a very limited sample preparation. Proton spectroscopy dominates NMR-based metabonomics for sensitivity reasons. The identification of metabolites is achieved by comparing resonances observed in the complex biological matrices with reference spectra from libraries or by spiking experiments with pure compounds. Nevertheless, the limited spectral dispersion of proton NMR (^{1}H) spectroscopy, mostly 12 ppm, results in the acquisition of highly overlapped metabolite signals. Multi-dimensional NMR techniques can be applied to resolve some of the overlapped signals and identify molecules [8,9]. Furthermore, statistical techniques based on the exploitation of the co-linearity between NMR signals can be applied for the identification of compounds in complex mixtures when series of identically registered profiles are available [10,11]. However, carbon-13 (^{13}C) spectroscopy is sometimes mandatory for the unambiguous assignment of NMR signals. Although technological advances in the cryogenic probes have significantly improved the observation of ^{13}C resonances, preliminary enrichment or purification steps, although time consuming, are often mandatory to establish structural identity of low concentration and unknown metabolites. Such sample preparation needs to be rapid, robust and reliable for a wide range of molecular structures to comply with the holistic and high throughput features of metabonomics.

Abbreviations: HPLC, high-pressure liquid chromatography; NMR, nuclear magnetic resonance; SPE, solid phase extraction.

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* Corresponding author. Tel.: +41 21 785 91 65; fax: +41 21 785 94 86.
E-mail address: serge.rezzi@rdls.nestle.com (S. Rezzi).

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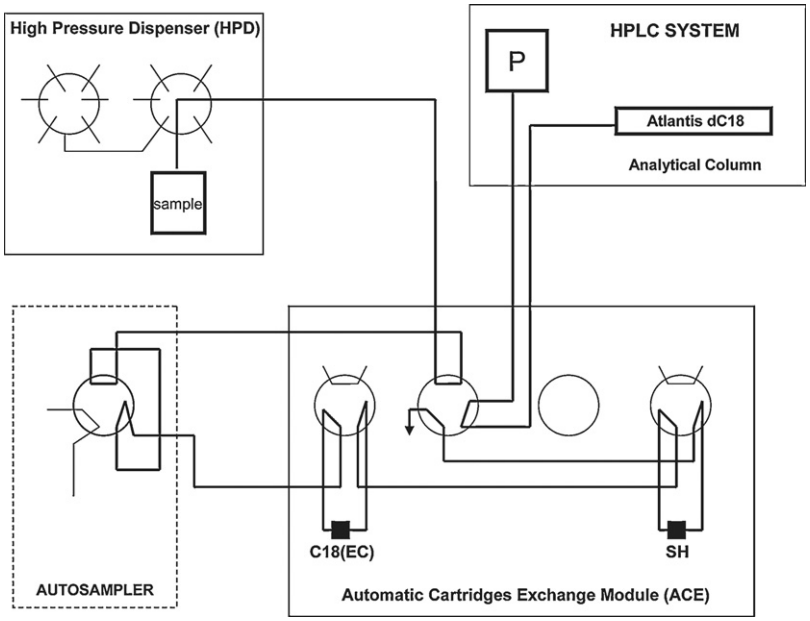

Fig. 1. Schematic diagram of the SPE-RP-HPLC system for automated sample fractionation. During the extraction mode both cartridges are placed in a loop with a switching valve. The cartridges are washed and conditioned with solvent from the high-pressure dispenser. An aliquot of the sample is loaded onto the SPE cartridges. The metabolites are adsorbed first on the C_{18} cartridge. The C_{18} non-retained analytes are trapped on the SH cartridge. The waste is recovered to analyze non-adsorbed metabolites. The SPE cartridges are then eluted separately onto the analytical column with the RP-HPLC mobile phase. First, the left valve is turned to flush the analytes from the C_{18} cartridge onto HPLC column. After the HPLC run, the left valve returns to the extraction mode, whilst the right valve is turned to flush the second SH cartridge onto the analytical column. The overall HPLC elution time lasts 44 min for each SPE cartridge.

Solid phase extraction (SPE) chromatography was previously combined to off-line NMR spectroscopic analysis (SPEC-NMR) for the identification of specific classes of compounds, e.g. xenobiotic metabolites, in urine samples [12–14]. However, the SPEC-NMR method is based on the manual fractionation of biofluid samples using a single SPE cartridge followed by ^{1}H, ^{13}C and ^{19}F NMR spectroscopy. The on-line hyphenation of HPLC, SPE and NMR spectroscopy (HPLC-SPE-NMR) offers an interesting technical solution for the analysis of compounds from complex mixtures [15,16]. In this setup, SPE is placed after the chromatography to trap specific molecules on cartridges prior to NMR spectroscopy. In particular, the HPLC-SPE-NMR system allows to perform multiple SPE trappings, i.e. after several chromatographic cycles, which facilitates NMR spectroscopic analysis, especially when using cryogenic probes [17].

In this contribution, we introduce an analytical methodology based on the on-line coupling of SPE with reversed phase HPLC (SPE-RP-HPLC) combined to NMR spectroscopy to improve metabolite identification. We show the usefulness of this approach with the analysis of the metabolic composition of human and cat urine samples and NMR assignment of metabolites.

2. Experimental

2.1. Urine sample collection

A morning spot human urine sample was collected from a healthy male volunteer at Nestlé Research Center (Lausanne, Switzerland). The cat urine was sampled after 16 h of food deprivation in the frame of a nutritional metabonomic study conducted at Nestlé Purina (St. Louis, USA). All samples were collected under appropriate national legislation and guidelines. Urine samples were stored at −40 °C prior to analysis.

2.2. Solid phase extraction analysis

Urine samples were freeze-dried and reconstituted in 4 mL of the original sample resulting in a concentration factor of around 10 times. Concentrated samples were adjusted to pH 3.0 with a solution of hydrochloric acid 1 M (Fluka, Switzerland) prior to loading onto SPE cartridges. The SPE was performed at room temperature using a Symbiosis™ system double clamps (Spark Holland, The Netherlands) equipped with HySphere C_{18}-(EC) (40–90 μm, 2 mm × 10 mm) and HySphere Strong Hydrophobic (SH) resin (polystyrene–divinylbenzene, 15–25 μm, 2 mm × 10 mm) cartridges (Spark Holland) (Fig. 1). The C_{18} SPE cartridge ensures the selective trapping and preconcentration of non-polar metabolites. Therefore, the metabolites not selectively retained on the C_{18} cartridge can be trapped on the SH resin, which is particularly efficient for phenols and polar analytes. SPE cartridges were first preconditioned with 2 mL of acidified methanol (pH 3.0), and equilibrated with 3 mL of acidified water (pH 3.0). A volume of 400 μL of 10 times pre-concentrated urine (pH 3.0) was sequentially loaded onto the C_{18} and SH SPE cartridges. The valve was then switched to elute first the C_{18} cartridge using the HPLC solvent gradient onto an analytical column (Atlantis dC_{18} RP column 5.0 μm, 4.6 mm × 250 mm i.d.; Waters, Milford, MA, USA). Following the elution of the first cartridge, the second cartridge (HySphere SH) was subjected to a similar elution process. The SPE Symbiosis™ system was controlled using SPARKLINK software version 3.1 (Spark Holland).

2.3. RP-HPLC analysis

The separation of metabolites was performed using a Waters separation module (Alliance Waters 2695) equipped with a column heater (Alliance), a reversed-phase analytical column (Atlantis dC_{18}, 5.0 μm, 4.6 mm × 250 mm i.d.; Waters), a UV detector (model 2487, Waters) set at 210 nm, and a fraction collector (model F:C:II,

Waters). The separation was achieved in 44 min at 30 °C with a flow rate of 1 mL min^{-1}. The mobile phases consisted of acidified water (A, pH 3.0, Merck, Germany) and methanol (B, pH 3.0, Merck) using a gradient elution of 0–0% B at 0–5 min; 0–10% B at 5–11 min; 10–60% B at 11–26 min; 60–100% B at 26–32 min; 100–100% B at 32–40 min; 100–0% B at 40–44 min. Individual fractions following elution of both C_{18} and SH SPE cartridges were collected every 1 and 4 min interval for human and cat urine, respectively. The collected fractions were evaporated under nitrogen and kept at −40 °C. Chromatography system was controlled using Waters Empower software (Waters).

2.4. ^{1}H and ^{13}C NMR spectroscopy

The SPE-RP-HPLC fractions were reconstituted in deuterated (Euriso-top, France) phosphate buffer solution (Na_2PO_4 and Na_2HPO_4, final concentration of 0.2 M) containing 1 mM of sodium-3-(trimethylsilyl)-[2,2,3,3-$^{2}H_4$]-1-propionate (TSP, Armar Chemicals, Switzerland) for chemical shift calibration. The samples were centrifuged at 17000 × *g* for 10 min and transferred into 1 mm NMR tubes.

^{1}H and ^{13}C NMR spectra were acquired for each sample on a Bruker Avance II 600 MHz NMR spectrometer (Bruker Biospin, Rheinstetten, Germany) using inverse 1 mm and 1.7 mm probes. The compositional study of human urine was completed using an inverse 5 mm cryogenic probe.

For each fraction, a proton spectrum was acquired using standard one-dimensional pulse sequence (D1-90°-t1-90°-tm-90°-acquisition), with water suppression during the relaxation (2 s) and mixing time (100 ms) delays. The 90° pulse length was adjusted on each sample and 128–512 transients were collected into 64 K data points using a spectral width of 12019.2 Hz. The free induction decays were multiplied by an exponential function equivalent to a line-broadening of 1 Hz prior to Fourier transformation. The acquired NMR spectra were manually phased and baseline corrected, and referenced to the methyl resonance of TSP at δ 0.00.

For assignment purposes, homo- and heteronuclear two-dimensional spectra were acquired on selected samples: ^{1}H–^{1}H correlation spectroscopy (COSY) [18], total correlation spectroscopy (TOCSY) [19], nuclear Overhauser enhancement spectroscopy (NOESY) [20], ^{1}H–^{13}C heteronuclear single quantum correlation (HSQC) [21], and heteronuclear multiple bond correlation (HMBC) [22].

For COSY, 256 increments with 32 transients per increment were collected into 2 K data points. The TOCSY NMR spectra were acquired with 32 transients per increment, 512 increments collected into 4 K data points, and using the MLEV-17 spin-lock scheme for ^{1}H–^{1}H transfers with a spin-lock mixing time of 60 ms. The NOESY NMR spectra were acquired with 48 transients, 400 increments collected into 4 K data points and with a mixing time of 150 ms. The relaxation delay between successive pulse cycles was 1.5 s for all pulse sequences. The spectral width in both dimensions was 7000 Hz. The sine-bell (COSY) and sine-bell squared (TOCSY, NOESY) window functions were applied prior to Fourier transformation.

The HSQC NMR spectra were recorded with gradient selection and shaped pulses. Spectra were acquired with 128 transients per increment, 256 increments collected into 2 K data points and a spectral width of 9600 and 30000 Hz in the first and second dimensions, respectively. The HMBC spectra were recorded with gradient selection, and low pass filtering. Spectra were acquired with 256 transients per increment, 512 increments collected into 4 K data points, and a spectral width of 9600 and 36000 Hz in the first and second dimensions, respectively. The coupling constants for HSQC and HMBC were set to 145 and 8 Hz, respectively. The sine-bell (HMBC) and sine-bell squared (HSQC) window functions were applied prior to Fourier transformation. The processing of 1D and 2D NMR spectra and the identification of metabolites using automatic matching routines were achieved using Topspin 2.0 and Amix 3.7.10 software packages (Bruker Biospin), respectively.

3. Results and discussion

3.1. Optimum SPE-RP-HPLC setup for urine fractionation

SPE is widely employed as preparation technique to purify specific compounds or sets of molecules. Very often, *a priori* knowledge of physicochemical properties of targeted compounds is mandatory to select appropriate stationary and elution phases. However, a complete fractionation of a biofluid such as urine requires the use of different SPE cartridges with complementary retention properties to address the molecular diversity of metabolites (polarity and molecular mass).

Our methodology associates a pre-concentration of urinary metabolites on SPE cartridges with an analytical RP-HPLC separation (Fig. 1) in fully automated mode. During the extraction mode both cartridges are placed in a loop with a switching valve. The cartridges are washed and conditioned with solvent from the high-pressure dispenser. The metabolites are adsorbed first on the C_{18} cartridge, the non-retained analytes being further trapped on the SH cartridge. The waste is recovered to analyze non-adsorbed metabolites. The SPE cartridges are then eluted separately onto the analytical column with the RP-HPLC mobile phase. The use of HySphere C_{18} and SH SPE cartridges ensures the retention of a wide range of non-polar and polar metabolites, respectively. Moreover, the fully automated Symbiosis™ system warrants optimal inter-runs repeatability.

3.2. SPE-RP-HPLC NMR spectroscopic analysis of human urine

The urine fractions were collected at 1 min intervals in dark bottles, dried overnight under nitrogen, and reconstituted in deuterated NMR buffer. A total of 72 metabolites could be identified by NMR spectroscopy by matching the 1D and 2D spectra of fractions with spectra of pure compounds in public and in-house libraries (Tables 1 and 2). To date, our NMR spectra database contains almost 500 metabolites (unpublished data) for which series of 1D and 2D spectra were registered at different pH values to cover the range of signal variations in biofluid analysis. The actual cartridge combination provides a complementary trapping of alcohols, organic acids and derivates, aromatic acids, purines and pyrimidines, amino acids and derivates, vitamins, sugars, amines, and alkaloids. On the 72 identified metabolites, 28 and 23 compounds were specifically retained on the C_{18} and SH cartridges, respectively, whereas 21 metabolites were common in both trapping techniques (Tables 1 and 2). Metabolites such as creatinine, hippurate, phenylacetylglutamine, and citrate for instance were identified in several fractions as a result of their high concentration in urine (Tables 1 and 2). Interestingly, SPE-RP-HPLC enables efficient separation and enrichment of different aromatic compounds such as 3-indoleacetate, 4-aminobenzoate, hippurate, phenylacetylglutamine, 3,4-dihydroxyphenylacetate, 4-hydroxyphenyllactate. Several metabonomic studies probing mammalian metabolism have suggested that some of these aromatic compounds are the result of metabolic activity of gut microflora [23–25]. The SPE-RP-HPLC methodology is therefore a valuable separation tool to aid unambiguous identification of microbial metabolites in complex biofluids. However, despite the metabolite enrichment provided

Table 1
Human urine metabolites eluted from HySphere C_{18} cartridge onto the Atlantis dC_{18} analytical column

20	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	Fractions collected between 4-20 min * HySphere C_{18}
															*	*	Alanine
															*	*	Threonine†
																*	Glutamine
																*	Histidine†
														*	*		Valine
															*		*N*,*N*-dimethylglycine
																*	Phtalate
									*	*	*	*	*				Nicotinate
									*				*				Creatinine†
									*	*			*				Citrate
											*	*					Oxypurinol
												*					Uracil†
											*	*					Pseudouridine†
												*					D-Fucose
												*					1,2-propanediol
								*	*	*	*						2-aminopropanol†
											*						Ascorbate
									*	*	*						Cis-acotinate
											*						L-Leucine†
			*	*				*	*	*							Isobutyrate†
										*							Urocanate
									*								4-hydroxyphenyllactate†
								*	*								Tyrosine†
						*											Diglycolate
						*											Glycolate†
						*											Fumarate†
						*											Anserine
					*												3-hydroxyisovalerate†
				*	*												Trimethylamine-*N*-oxide
				*	*												Betaine
				*													2-aminobutyrate
			*	*													Phenylalanine†
			*														6-methylnicotiamide
		*															Furoylglycine†
	*																3,4-dihydroxyphenylacetate
*																	Tiglyglycine
*																	*N*-isovaleroylglycine
*																	Indoxylsulfate†
													●	●	●		Hippurate†
													●	●	●		Phenylacetylglutamine†
												●					Pimelate
												●					Adipate†
												●					4-aminobenzoate
											●						Xanthosine†
										●							Norvaline†
										●							Dimethylurea
									●								Suberate
								●									3-indoleacetate
								●									*N*-acetyl-tryptophan
● *	● *	● *	● *	● *	● *	● *	● *	● *	● *	● *	● *	● *	● *	* ●	* ●	* ●	Unknown compounds
37	36	35	34	33	32	31	30	29	28	27	26	25	24	23	22	21	Fractions collected between 21 to 37 min ● HySphere C_{18}

Key: †Metabolites eluted from both C_{18} and SH SPE cartridges.

by this method numerous metabolites in the collected fractions remain unidentified. The actual method could be easily completed with the implementation of mass spectrometry analyses, e.g. high-resolution molecular mass determination and fragmentation analysis, to improve the identification capacity. For instance, MS measurements are particularly efficient for the unambiguous structural determination of sulphated metabolites, since this kind of conjugation does not introduce further NMR-detectable nuclei [26].

3.3. Application of SPE-RP-HPLC to the NMR spectroscopic analysis of cat urinary metabolites

We present the SPE-RP-HPLC methodology as an efficient tool to reduce the characterization time of unassigned metabolic biomarkers in the context of a metabonomic study on cat metabolism. Feline urine samples are marked by the presence of specific metabolites when compared to samples from human subjects [27], dogs

Table 2
Human urine metabolites eluted from HySphere SH cartridge onto the Atlantis dC_{18} analytical column

20	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	Fractions collected between 4-20 min * HySphere SH
																*	D-glucose
														*	*		1-methylnicotinamide
			*	*	*	*	*	*	*	*	*	●	*	*	*		Creatinine†
															*		Cytosine
															*		3-aminoisobutyrate
														*			Trigonelline
											*	*					Uracil†
												*	*				Pseudouridine†
											*						L-leucine†
											*						2-methylglutarate
											*						2-aminopropanol†
											*						Threonine†
											*						Isonicotinate
										*							Isobutyrate†
										*							3-hydroxy-4-methoxymandelate
										*							Pyridoxine
					*	*	*	*	*								Tyrosine†
						*											Fumarate†
						*											Trans-aconitate
					*												Histamine
					*												Histidine†
					*												Thymine
					*												Succinate
					*												Pyruvate
			*														Phenylalanine†
			*														Xanthosine†
			*														3-hydroxyisovalerate†
			*														Isobutyrylglycine
*																	Tropate
*																	Furoylglycine†
*																	Homovanilate
															●		4-aminohippurate
															●		Adipate†
			●							●	●	●	●	●			Hippurate†
														●			Indoxylsulfate†
														●			Norvaline†
													●				Isocaproate
																	4-hydroxyphenyllactate†
													●				Phosphothreonine
													●				2-oxoisocaproate
										●	●	●					Phenylacetylglutamine†
												●					Glycolate†
											●						4-hydroxyphenylacetate
●	●																Lactate
● *	● *	● *	● *	● *	*	● *	● *	● *	● *	● *	● *	● *	● *	● *	● *	● *	Unknown compounds
37	36	35	34	33	32	31	30	29	28	27	26	25	24	23	22	21	Fractions collected between 21 to 37 min ● HySphere SH

Key: †Metabolites eluted from both C_{18} and SH SPE cartridges.

[28] or rodents [6,23]. In particular, the NMR urine profile showed complex metabolite patterns in the aromatic area and resonances at 1.35 and 1.86 ppm that were not assigned. A quick analysis of all the collected fractions from both SPE cartridges with 1D ^{1}H NMR spectroscopy showed that these metabolites were efficiently trapped by the C_{18} SPE cartridge and further separated by analytical HPLC (Fig. 2). For instance, main aromatic resonances were attributed to 4-hydroxyphenylacetate, 4-hydroxyphenyllactate, 4-cresol derivate, hippurate, inosine, indole-3-lactate and phenylacetylglycine using 1D and 2D NMR spectroscopy. In addition, the spin system containing the unassigned 1.35 and 1.86 ppm resonances was isolated in the fraction 2 (4–8 min). Two SPE-RP-HPLC cycles were sufficient to accumulate the amount of metabolite required to successfully acquire heteronuclear 2D NMR HSQC and HMBC experiments. Two-dimensional homonuclear (COSY, TOCSY, NOESY) and heteronuclear (HSQC and HMBC) experiments were carried out to establish ^{1}H–^{1}H and ^{1}H–^{13}C spin connectivities (Figs. 3 and 4, Table 3). The interpretation of NMR spectra enabled the assignment of resonances to 2-amino-7-hydroxy-5,5-dimethyl-4-thiaheptanoic acid or felinine (Table 3, Fig. 3A). The integration of the signals in the proton NMR spectrum gives the following intensity ratios 1:2:2:2:6, and corresponds to a total of 13 protons (Fig. 3B). The proton signals were attributed as the following: 3.94 ppm (dd),

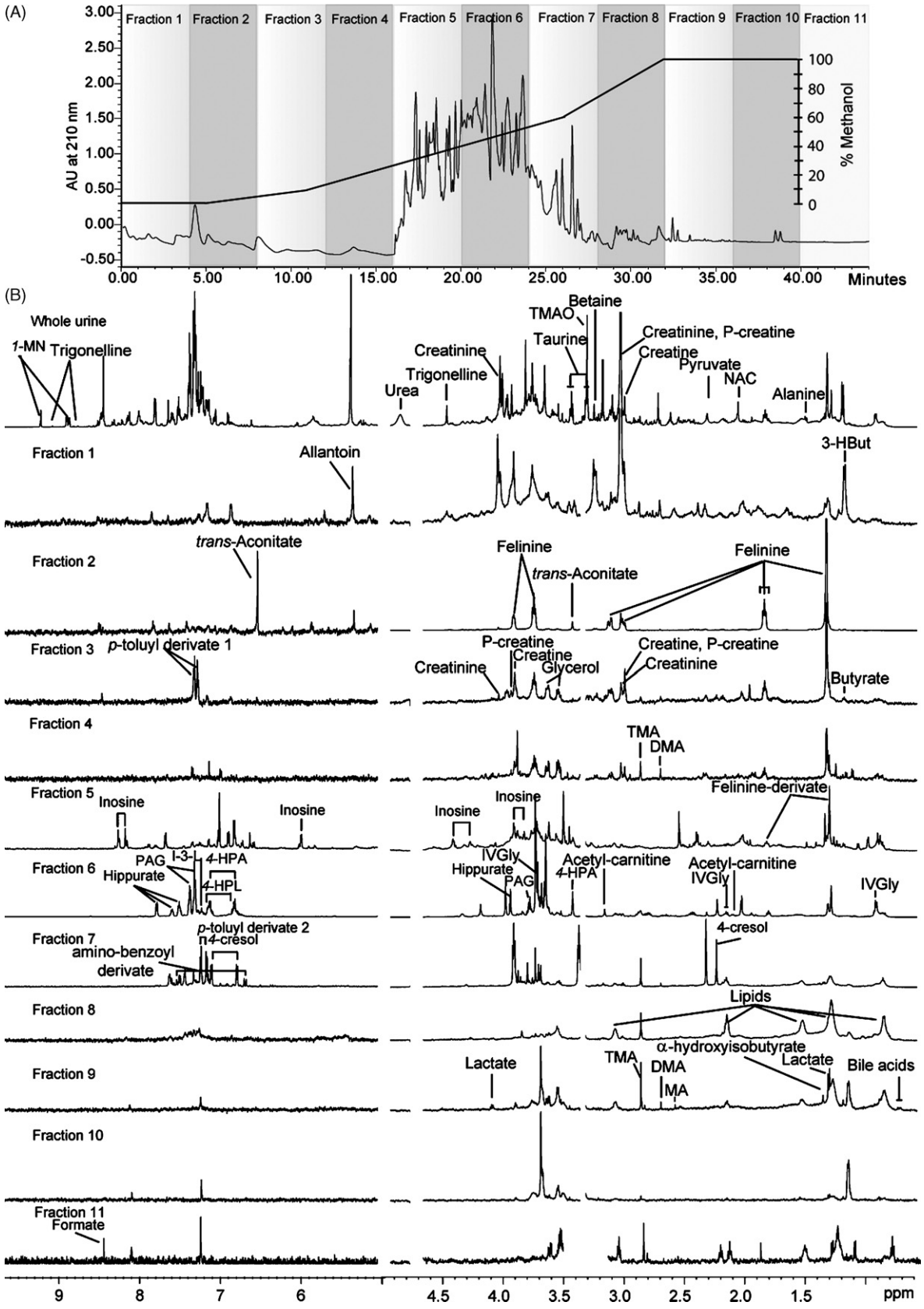

Fig. 2. Compositional analysis of cat urine. UV chromatogram of the HPLC elution of the first C_{18} cartridge (A) and 600 MHz ^{1}H one-dimensional spectra of the eluted fractions collected at 4 min intervals (B). The scale of spectra in the aromatic region (δ 5.0–9.5) was expanded for better visualization.
Key: 1-MN, 1-methylnicotinamide; 3-Hbut, 3-D-hydroxybutyrate; 4-cresol, 4-cresol derivate; 4-HPA, 4-hydroxyphenylacetate; 4-HPL, 4-hydroxyphenyllactate; DMA, dimethylamine; I-3-L, indole-3-lactate; IVGly, isovalerylglycine; MA, methylamine; NAC, *N*-acetyl-glycoproteins; PAG, phenylacetylglycine; P-creatine, phosphocreatine, TMA, trimethylamine; TMAO, trimethylamine-*N*-oxide.

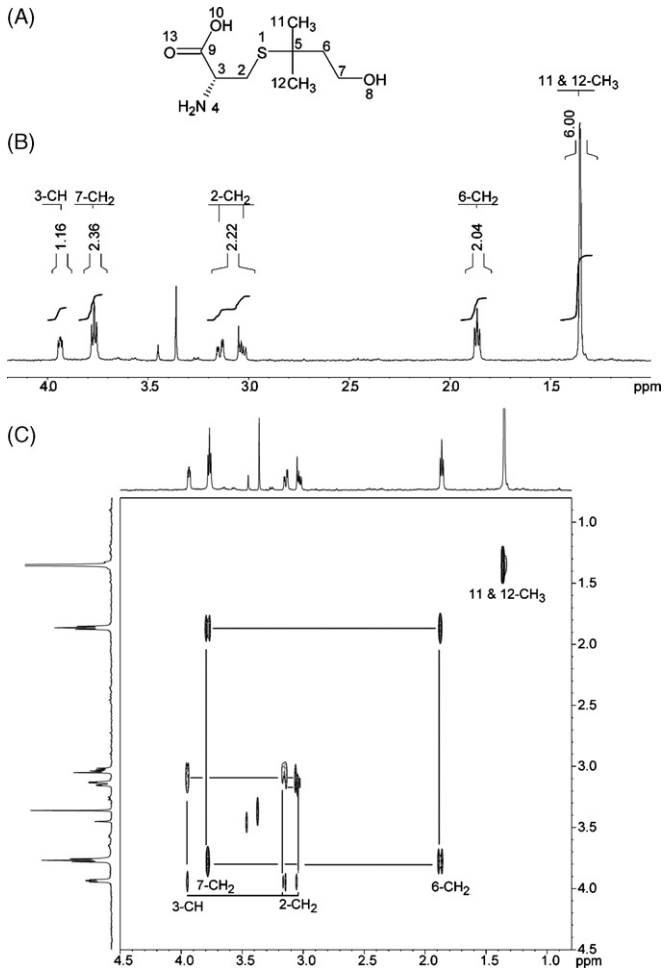

Fig. 3. NMR assignment of felinine. Chemical structure of felinine (A), 600 MHz ^{1}H one-dimensional (B) and two-dimensional TOCSY (C) NMR spectra of a felinine-enriched fraction from cat urine.

Table 3
600 MHz ^{1}H and ^{13}C NMR data of felinine (2-amino-7-hydroxy-5,5-dimethyl-4-thiaheptanoic acid) in D_2O with TSP as an internal standard, at 300 K

Nuclei	^{1}H NMR	*J* (Hz)	^{13}C NMR
2-CH_2	3.15 ppm (dd)	4.28, 13.76 Hz	31.10 ppm
	3.03 ppm (dd)	7.35, 13.76 Hz	
3-CH	3.94 ppm (dd)	4.28, 7.35 Hz	57.00 ppm
4-NH_2	Not identified		
5-C			47.80 ppm
6-CH_2	1.86 ppm (t)	6.89 Hz	45.90 ppm
7-CH_2	3.77 ppm (t)	6.89 Hz	61.80 ppm
8-OH	Not identified		
9-COOH	Not identified		175.78 ppm
11-CH_3*	1.353 ppm (s)		31.04 ppm
12-CH_3*	1.349 ppm (s)		

Shift values are given in ppm from internal TSP standard, coupling constant *J* in Hz. Multiplicity abbreviations for ^{1}H NMR: s, singlet; dd, doublet of doublet; t, triplet. *: chemical shifts could be inverted.

methine group (3-CH); 3.77 ppm (t), methylene group (7-CH_2); 3.15 ppm (dd)–3.03 ppm (dd), methylene group (2-CH_2); 1.86 ppm (t), methylene group (6-CH_2); 1.353 ppm (s) and 1.349 ppm (s), two methyl groups (11-12-CH_3). The TOCSY spectrum showed cross peaks of two individual spin systems from the 3-mercapto-3-methylbutan-1-ol and cysteine moiety (Fig. 3C). In addition, 2D NOESY spectrum exhibited a spatial interaction between the methylene group (2-CH_2) of the cysteine and methyl groups (11-12-CH_3) of the 3-mercapto-3-methylbutan-1-ol moieties (data not shown). These results were further confirmed by assigning direct proton–carbon couplings in the molecule: 1.353–1.349 ppm with 31.04 ppm; 3.94 ppm with 57.00 ppm; 3.77 ppm with 61.80 ppm; 3.15–3.03 ppm with 31.10 ppm; 1.86 ppm with 45.90 ppm (data not shown). Finally,the HMBC spectrum enabled the assignment of the carbons C9 of the carboxylic group at 175.78 ppm and the quaternary carbon C5 at 47.80 ppm (Fig. 3A) from the cross peaks with protons of methine at 3.94 ppm (3-H), methyl at 1.353 and 1.349 ppm (11-12-H), and methylenes at 1.86 ppm (6-H), 3.15 and 3.03 ppm (2-H), and 3.77 ppm (7-H). In addition to felinine, the SPE-RP-HPLC allowed identifying various amino acids, organic acids, osmolytes, polyols, methylamines, saturated and unsaturated short and medium-chain fatty acids and their derivates, ketone bodies, *N*-acetyl-glycoproteins, aromatic compounds, and bile acids.

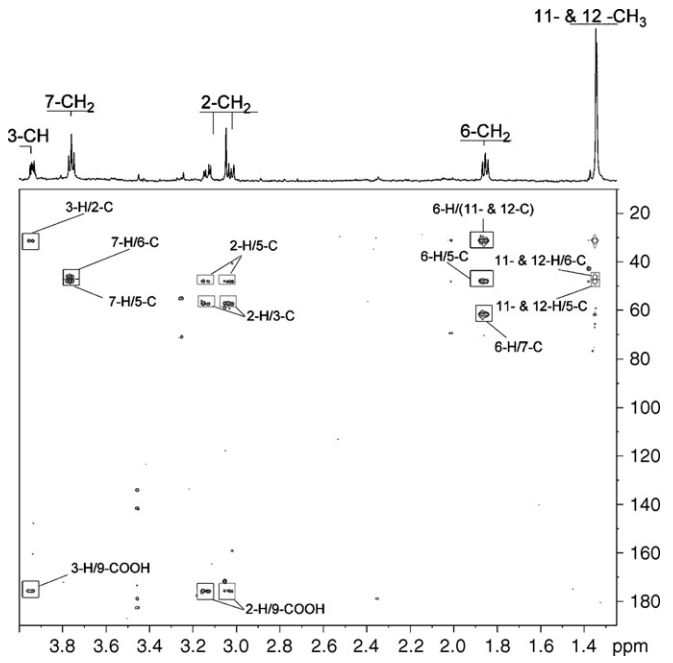

Fig. 4. 600 MHz ^{1}H–^{13}C two-dimensional HMBC NMR spectrum of the cat urine fraction eluted from the first C_{18} cartridge onto the Atlantis dC_{18} analytical column.

4. Conclusions

SPE-RP-HPLC methodology offers an efficient and time-saving solution for the identification of metabolites in complex mixtures. We show data on the enrichment and separation of low molecular weight metabolites with various polarities. In combination with a robust spectral/mass database, it is a powerful tool for the compositional study of biological matrices and the characterization of metabonomic biomarkers. The usefulness of this approach was demonstrated by the analysis of human and cat urine metabolomes using NMR spectroscopy. However, this fractionation method can be easily coupled to mass spectrometry with or without direct hyphenation. Applied to metabonomics, such an approach is promising to identify endogenous compounds, mammalian gut microbial co-metabolites, drugs and their metabolites as well as food-related compounds present at very low concentration in various liquid matrices.

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