Electronic Supplementary Material (ESI) for Analyst.

Supporting Information

NMR detection in biofluid extracts at sub-μM concentrations via *para*-H_2 induced hyperpolarization.

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Chemicals and materials

HPLC grade methanol for SPE cartridge activation was obtained from Thermo Fischer Scientific. Water for cartridge conditioning and washing was purified in house by a Millipore Milli-Q water purification system. Methanol-d_4 for extraction and NMR sample preparation and D_2O were obtained from Cambridge Isotope Laboratories. Nicotinamide (NA) was obtained from Sigma-Aldrich. N,N-Diethylnicotinamide (dENA, nikethamide) and N-ethylnicotinamide (mENA) were obtained from TCI Europe. The polarization transfer catalyst precursor, [Ir(Cl)(COD)(IMes)], was synthesized according to earlier published methods.[1] The co-substrate 1-methyl-1,2,3-triazole (mtz) was prepared by an earlier method.[2] Solid Phase Extraction (SPE) cartridges were obtained from Waters (Oasis HLB, 30 µm particle size, 200 mg sorbent bed, 6 mL cartridge volume) and used according to manufacturer guidelines. All sample preparation procedures were carried out under air atmosphere. The densities of all methanolic extracts and solutions were assumed to be equal to methanol-d_4 at 0.888 g/mL.

NMR experiments were carried out on a 600 MHz Bruker Avance III spectrometer equipped with a cryo-cooled HCN probe and on a Varian Unity Inova 500 MHz spectrometer equipped with a cryo-cooled HCN probe. All chemical shifts are referenced to residual solvent peaks. *p*-H_2 was prepared by cooling a 2 L pressure vessel charged with 40 bar of hydrogen (purity 5.0) to 77 K in the presence of activated charcoal. The resulting 51% *p*-H_2 was loaded into an aluminum cylinder for storage and transportation.

Urine sample handling

Samples for method development were collected by a healthy volunteer (male) as morning first mid-stream urine. Samples used in the repeatability study were collected by 5 volunteers (all male) as mid-stream urine, without any control over the time of day for sample collection (to emulate a more realistic scenario in doping detection). Samples were divided into 12 ml aliquots and frozen at -20 °C.[3] Individual aliquots were thawed over a room temperature water bath when needed.

Since the pH of samples can have a noticeable impact on sample preparation and exercise is known to cause acidosis,[4] pH of urine aliquots was adjusted to 7.5 with dropwise addition of 1 M NaOH right after thawing. pH 7.5 urine was centrifuged at room temperature for 15 min (3750 RCF) to remove sediment and decanted to a clean container. When required, urine was gravimetrically spiked with 1 mM stock solutions of dENA and mENA, mixed and used for SPE. The SPE protocol is described in the Experimental Section of the article main text.

NMR sample preparation

130 mg of a catalyst precursor stock solution consisting of 4.8 mM of [Ir(Cl)(COD)(IMes)] and 72 mM of mtz co-substrate in methanol-d_4 was weighed into a 5 mm medium wall Wilmad® quick pressure valve NMR tube. The tube was vacuumed briefly, pressurized under 5 bar of H_2, shaken vigorously and allowed to hydrogenate (activate) at room temperature for 2 h to generate a solution of 4.8 mM $[Ir(H)_2(IMes)(mtz)_3]^+$ polarization transfer catalyst in the presence of excess mtz.

The tube with the activated catalyst solution was opened and 390 mg of SPE extract was added. The resulting mixture was quickly attached to the experimental setup[5] and *p*-H_2 was bubbled through the sample for a few seconds to saturate it. No sample degassing (as in traditional SABRE experiments) is necessary since *p*-H_2 bubbling during the experiment serves to deoxygenate the sample and the possibly remaining dissolved oxygen does not cause noticeable extra relaxation during the time scale of the high field experiment.[5]

NMR experiments

An adaption of a SEPP pulse scheme (Figure S2)[6,7] was used to record the hyperpolarized hydride spectra in Figure 2 of main text. In general, hydride signals *trans* to aromatic six membered nitrogenous heterocycles appear on the upfield side of the hydride spectrum (δ < -21.8 ppm) and hydride signals corresponding to five membered nitrogenous heterocycles (including mtz) appear on the downfield side of the spectrum (δ > -21.8 ppm). The hydride doublet *trans* to nicotinamide (NA) was assigned by spiking of the sample.

An adapted version of an earlier published 2D quantitative *p*-H_2 induced polarization method[5] was used in the filtered 1D NMR experiment. The overlapping hydride signals of NA, dENA and mENA at -22.96 ppm where selectively excited with a 175 Hz wide band selective excitation pulse. The excitation bandwidth had to be at least 175 Hz to ensure uniform excitation of all three hydride signals of interest. This bandwidth will also cause partial excitation of 2 more endogenic metabolites at -23.1 ppm (see main text Fig. 4 & Fig. 5), which, however, causes no overlap with the compounds of interest.

51% enriched *p*-H_2 was introduced into the sample at high field with a previously described experimental setup.[5] The NMR experiment consisted of 8 dummy scans to saturate the solution with *p*-H_2 followed by 64 scans, each consisting of 1.2 s *p*-H_2 bubbling time, 0.7 s backpressure and line shape recovery delay, the pulse sequence and 1.2 s acquisition time. The FID was Fourier transformed into 8k data points while applying a sine squared window function with a 80 degree sine bell shift. All spectra are displayed and analyzed in magnitude mode and peak areas obtained with uniform integration limits across all datasets. All samples were subjected to 3 measurements and the results (peak integrals) are presented as averages with standard deviations.

Repeatability

A volume of 28.9 mL of urine was spiked to 5 µM of dENA and mENA with 1 mM stock solutions. Four 5 mL aliquots of the spiked urine were subjected to SPE and NMR. Integrals in Table S1 reflect the averages of arbitrarily normalized peak integrals of 3 repeated measurements of each sample with corresponding standard deviations. The Average column reflects the averages of all 12 measurements.

Table S1. Analysis of 4 identically prepared urine samples.

	Sample 1		**Sample 2**		**Sample 3**		**Sample 4**		**Average**		
	Integral (a.u.)	Stdev	Integral (a.u.)	Stdev	Integral (a.u.)	Stdev	Integral (a.u.)	Stdev	Average	Stdev	RSD
dENA	0.959	0.026	1.004	0.085	0.853	0.022	1.044	0.022	0.965	0.085	8.8%
mENA	0.694	0.053	0.727	0.046	0.700	0.067	0.764	0.037	0.721	0.053	7.3%

In a separate experiment 12 mL urine samples from 5 different persons were gravimetrically spiked to 2 µM of dENA and mENA by using 1 mM stock solutions. 5 mL aliquots of each sample were subjected to SPE and NMR. Integrals in Table S2 reflect the averages of arbitrarily normalized peak integrals of 3 repeated measurements of each sample with corresponding standard deviations. The Average column reflects the averages of all 15 measurements.

Table S2. Analysis of 5 urine samples prepared identically from urine of 5 different persons.

	Sample 1		**Sample 2**		**Sample 3**		**Sample 4**		**Sample 5**		**Average**		
	Integral (a.u.)	Stdev	Integral (a.u.)	Stdev	Integral (a.u.)	Stdev	Integral (a.u.)	Stdev	Integral (a.u.)	Stdev	Average	Stdev	RSD
dENA	0.964	0.021	0.956	0.020	0.956	0.020	0.912	0.006	0.974	0.046	0.968	0.052	5.4%
mENA	1.209	0.059	1.199	0.058	1.038	0.038	0.898	0.056	1.164	0.056	1.106	0.134	12.1%

Calibration for quantitative analysis

The linearity of the signal response and the integrals to analyte concentration in urine (main text, Figure 6) was established by gravimetrically spiking 6 mL aliquots of urine to increasing concentrations (0.5, 2.0, 5.0, 10.0 and 25.0 µM) of dENA and mENA with 1 mM stock solutions prior to SPE. 5 mL of each spiked aliquot was subjected to SPE and NMR. The averages of 3 parallel measurements of each sample were fitted with linear regression analysis, giving a calibration curve on Fig 5 of main text. This method also calibrates for the recovery of the analytes by SPE.

Table S3. Calibration with urine spiking before SPE.

	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		Fit				
	Spiking Integral (a.u.)	0.5 µM Stdev	Spiking Integral (a.u.)	2 µM Stdev	Spiking Integral (a.u.)	5 µM Stdev	Spiking Integral (a.u.)	10 µM Stdev	Spiking Integral (a.u.)	25 µM Stdev	Slope	Stdev	Intercept	Stdev	R^2
dENA	0.323	0.009	1.028	0.063	2.194	0.143	4.184	0.553	11.161	0.868	0.4414	0.0091	0.0258	0.1113	0.9987
mENA	0.430	0.430	1.212	0.082	2.977	0.368	5.815	0.663	-	-	0.5699	0.0052	0.1154	0.0297	0.9998

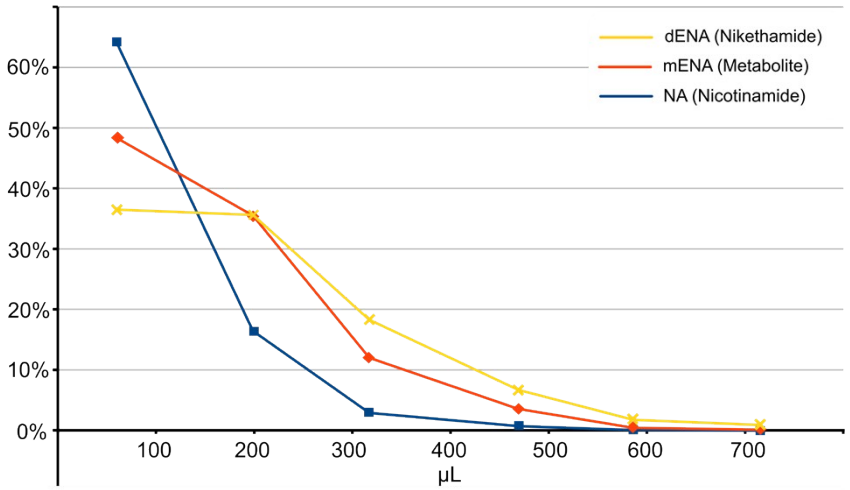

Figure S1. Release of analytes from Oasis HLB SPE cartridge after extracting from 5 mL of urine spiked to 20 µM of each component. Each data point reflects the signal from an approx. 100 µL fraction, diluted to 500 µL with methanol-d_4 and mixed with preactivated catalyst mixture. All fractions were weighed and converted to fraction volume. Integrals were obtained from the NMR experiment as described above. Percentages on the ordinate correspond to % of signal from the total signal obtained across all fractions.

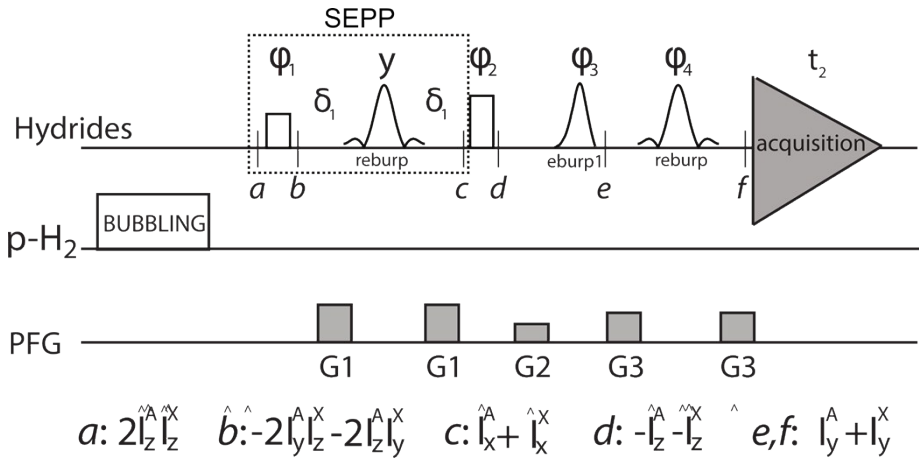

Figure S2. SEPP pulse scheme[6,7] to acquire a *p*-H_2 enhanced NMR spectrum of hydrides of complexes **1**, centered at -21.729 ppm. Rectangular pulses indicate a low-power 45-degree pulse and a low power 90-degree pulse, respectively. Shapes represent selective pulses with a bandwidth of 2000 Hz. Delay durations: δ_1 = 61.1 ms. Phase cycling: Φ_1: x, -x; Φ_2: 2(y), 2(-y); Φ_3: 4(x), 4(y), 4(-x), 4(-y); Φ_4: 4(y), 4(-x), 4(-y), 4(x); receiver: x,-x,-x,x,y,-y,-y,y,-x,x,x,-x, -y,y,y,-y.

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