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Quantification of caffeine in human saliva by Nuclear Magnetic Resonance as an alternative method for cytochrome CYP1A2 phenotyping

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ABSTRACT

The first step in caffeine metabolism is mediated for over 95% by the CYP1A2 isoform of cytochrome P450. Therefore, CYP1A2 activity is most conveniently measured through the determination of caffeine clearance. The HPLC quantification of caffeine is fully validated and is the most widely used method. It can be performed on saliva, which is gaining importance as a diagnostic biofluid and permits easy and low invasive sampling.

Here, we present a quantitative ^{1}H nuclear magnetic resonance (NMR) method to determine caffeine in human saliva. The procedure is simple because it involves only an ultra-filtration step and a direct extraction in a deuterated solvent, yielding a matrix that is then analyzed. The reliability of this NMR method was demonstrated in terms of linearity, accuracy, recovery, and limits of detection (LoD). Good precision (relative standard deviation, RSD $<4\%$), a recovery of $>95\%$ and LoD of $6.8 \cdot 10^{-7}$ mol L^{-1} were obtained. The method was applied to samples collected from different volunteers over 24 h following a single oral dose of about 100 mg of caffeine administered with either coffee beverage or a capsule.

1. Introduction

The enzyme cytochrome P450 1A2 (CYP1A2) accounts for about 13% of the total cytochromes P450 (CYPs) in the human liver [1] and is involved in a number of metabolic pathways that process endogenous substrates, xenobiotics and environmental toxins and also in the activation of carcinogens, such as dietary heterocyclic amines or polycyclic aromatic hydrocarbons [2,3]. It is responsible for the metabolism of several drugs used in various therapeutic areas, including antidepressants, antipsychotics, antiarrhythmics, broncho-dilators and many others [2].

Constitutional and genetic factors can cause different levels of CYP1A2 activity and factors such as life style (smoking, physical exercise), diet (caffeine, cruciferous vegetables, grilled meat, alcohol) as well as many drugs are known to modulate enzyme induction or inhibition [2,3]. Depending on the analytical method used, *inter* individual differences up to 60-fold can be found, and 5- and 15-fold variations are common [3].

The study of CYP1A2 activity is important to better understand the great *intra* and *inter* individual variability in response to therapeutic treatments. Some drugs influence the metabolization rate of xenobiotics and even their own clearance leading to a higher or lower exposition to the corresponding metabolites. In such cases, unpredictable CYP1A2 activity may cause non optimal therapeutic response or a bigger probability to develop adverse effects.

Considering the important role of CYP1A2 in the elimination or in the metabolism of a wide range of xenobiotics including drugs, environmental compounds and (pro) carcinogens [2,3], phenotyping represents the way to identify sources of variations of enzyme activity and, in turn, an instrument to calibrate drug therapies in the clinical practice, discover drug interactions and understand the cause of possible adverse drug effects or non-response to a therapy [3–5].

Usually, enzymatic activity can be easily determined through *ex vivo* tests in tissues expressing the enzyme, but this is not feasible in the case of CYP1A2 because this enzyme is not present in blood cells or in another readily accessible tissue or fluid. Since CYP1A2 is mainly confined to liver, to avoid liver biopsies, *in vivo* tests are preferred [3].

Caffeine is the most commonly used probe for CYP1A2 phenotyping as over 95% of the first step in its metabolism is mediated by CYP1A2, and it is ideal for many reasons, allowing non-invasive epidemiological studies: it is safe and commonly present in the diet, it can be administered orally and its gastrointestinal absorption is rapid and complete; once absorbed, it is found in all body fluids, there is no long-term accumulation and it is extensively metabolized by the liver with minimal renal elimination [3,5,6].

For CYP1A2 phenotyping, caffeine is the only substrate for which a fully validated method is available [3,5,6], and the

Abbreviations: DMF, N,N-dimethylformamide; HPLC, High Performance Liquid Chromatography; NMR, Nuclear Magnetic Resonance; qNMR, quantitative Nuclear Magnetic Resonance

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systemic clearance of caffeine, following the administration of a known dose, is considered the 'gold-standard' in comparison to CYP1A2 activity in liver biopsies [3,5]. The effectiveness of other proposed metrics is evaluated against this one: one example is the method based on the determination of the paraxanthine to caffeine ratio between 4 and 6 h after caffeine intake, that showed a good correlation too [3,5,7]. Literature studies on caffeine determination in various matrices (plasma, saliva, urine) are mainly based on HPLC (*High Performance Liquid Chromatography*) and the most widely used detection techniques are ultraviolet absorption [4,7–11] and to a minor extent mass spectrometry [12–16]. In most cases, a calibration curve is required for quantitative analysis. Some GC–MS methods have also been proposed (see for example [17]). With MS detection, the use of expensive stable-isotope labeled standards is common.

Biological matrices such as saliva and plasma are complex, principally because of the high content of proteins, and considering the low analyte concentrations expected in this type of studies (C_{max} = 1.2–1.9 μg mL^{-1} or 6–10 μmol L^{-1}), most of the methods proposed require a liquid–liquid extraction [7,8,10,14,16] or solid-phase extraction [9,11,12] step during the sample pretreatment, with subsequent solvent removal and reconstitution in an appropriate solvent.

In the present study, we analyzed caffeine clearance in saliva, which is the biofluid showing the closest correlation with immunoreactive CYP1A2 liver intrinsic activity [3]. In addition, saliva is an interesting matrix for drug monitoring purposes, gaining importance over plasma/serum, as it offers the advantage to be more easily, more cheaply, and less invasively sampled, ideal in the case of multiple and long treatments, especially in diseased individuals, children or elderly people [5,18].

This work describes the quantitative determination of caffeine in saliva by Nuclear Magnetic Resonance (NMR). A powerful characteristic of NMR spectroscopy is that it is inherently quantitative and it provides a linear response; because of these features, the lengthy construction of a calibration curve is usually not required and a reference compound (internal or external) is sufficient to provide absolute concentrations [19,20].

The method we propose is rapid because a filtration step allows the extraction to be performed directly in a deuterated solvent, with no need for solvent evaporation and reconstitution in a different solvent, involving a reduction of time of analysis and volume of solvent needed. For absolute quantification, an external standard in a coaxial insert is used which simplifies the sample preparation step and reduces the risk of introducing errors in the procedure.

The first part of the work was finalized to optimize sampling, pretreatment, and NMR protocol and included validation of the method in terms of precision and accuracy. The method was then applied to samples collected from volunteers to compare it to previously published studies.

2. Materials and methods

2.1. Chemicals

Capsules of caffeine (100 mg caffeine per capsule) were purchased from Scitec Nutrition P.P. Box 431975, Miami, FL33243, USA, distributed by Scitec KFT, Hungary. Caffeine (≥ 99.0%) was purchased from Sigma-Aldrich. As standard compound for quantitative determination of caffeine, N,N-dimethylformamide (DMF, ≥ 99.99% (GC), Fluka-Sigma-Aldrich) was used. Deuterated chloroform (99.96%D) was purchased from Sigma-Aldrich, and deuterated water (≥ 99.96% D), from Eurisotop. Ultrafiltration was performed using Sartorius Vivaspin devices (2 kDa cutoff).

2.2. Preparation of the standard solutions

Caffeine and DMF standard solutions were prepared weighing accurately the compound and the chosen solvent. In the case of the caffeine solution in water, considering the low solubility of caffeine in water (15 mg mL^{-1}), the exact concentration was verified using a UV calibration curve (λ_{max}=273 nm, ε=(9633.9 ± 70.4) L mol^{-1} cm^{-1}).

2.3. Participants and sample collection

Three healthy subjects (two females, one male), non smokers and not under pharmacological treatment were asked to abstain from any caffeine or methylxanthines intake for at least 48 h. Participants were asked to expectorate into a graduated conical disposable centrifuge tube (15 mL) a volume of about 5 mL of saliva corresponding to a collection time of approximately 3–6 min. Salivation was stimulated by chewing a piece of Parafilm®. At the beginning of the sampling day, a blank sample prior to caffeine ingestion was collected to assure the absence of the analyte. Samples were collected at least 30 min after any meal. The samples were stored at −20 °C. Caffeine was administered with a capsule (100 mg), assuring no permanence of caffeine in the mouth, or with an espresso coffee regular beverage (~6.5 g roasted coffee, 25 mL), in which the caffeine content was measured by ^{1}H NMR (~77 mg).

2.4. Sample treatment

The frozen saliva was thawed and centrifuged at 6000 rpm and the supernatant was ultrafiltered. Low-molecular-mass ultrafiltrates were obtained using Sartorius Vivaspin devices (2 kDa cutoff) washed with water before use. To 4 mL of filtered saliva, 1 mL of deuterated chloroform was added, the sample was vortex mixed (15 min) and then centrifuged (7200 rpm, 10 min, T=20 °C). The organic layer (500 μL) was transferred into a 5 mm precision glass NMR tube (Wilmad 535-pp) and the coaxial insert with the standard solution was put in the same tube, which was then analyzed.

Saliva was also analyzed as such or after the ultrafiltration step. In these cases, 10% of D_2O was added to the saliva, which was then analyzed in the same way.

2.5. NMR spectroscopy

^{1}H NMR data were acquired using a Bruker Avance DMX600 instrument, operating at 599.90 MHz and equipped with a 5 mm TXI xyz-triple gradient probe.

Aqueous ultrafiltered samples were acquired with the DPFGSE [21] sequence to maximize the sensitivity of the experiment, using an adiabatic pulse; for the $CDCl_3$ extracts, a common one pulse sequence was used. Typically, spectral widths of 6000 Hz, 32,768 data points were used. A relaxation delay of 43 s was used when the aliphatic spectral region was to be integrated and of 50 s when the aromatic region was to be integrated. The number of scans varied between 32 (25 min) and 128 (1 h 40 min) depending on caffeine concentration, to reach at least a S/N of 10. In the case of very low caffeine concentrations in the extracts (~0.75 μmol L^{-1}, 0.15 μg mL^{-1}), corresponding to 20–24 h after caffeine ingestion, and in the ultrafiltered aqueous samples, 256 scans were used (3 h 20 min).

The ACD software (ACD 12 Labs) was used to process the spectra. Fourier transformation was performed after zero filling the free induction decay data by a factor of 2 and after exponential line-broadenings of 0.2 Hz. Integrations were manually obtained after careful manual phase and baseline correction.

2.6. Quantitative determination of caffeine

To quantify absolute concentrations, an external standard was used: a coaxial insert (wgs-5bl, Wilmad Glass), filled with a DMF solution in D_2O, was placed inside the NMR tube. The use of DMF as an external standard has several advantages: DMF can be purchased very pure, it is a non volatile solvent soluble in D_2O, it is stable for a long time under the experimental conditions [22], and it gives signals both in the aliphatic region near the methyl signals of caffeine and in the aromatic region near the H8 proton of caffeine, in a free region of the NMR spectra. The concentration of the standard, $3 \cdot 10^{-4}$ mol L^{-1}, was determined gravimetrically.

The longitudinal relaxation time T_1 of all signals of interest was determined, using the inversion-recovery sequence, in the matrices, in the standard solutions, and in chloroform. The values determined are reported in Table 1.

To produce quantitative data, the relaxation delay was at least 5 times the longest measured T_1. If only the aliphatic region is considered, this corresponds to one of the methyl signals of DMF in D_2O, determining a relaxation delay of 43 s; if also signals in the aromatic region have to be considered, the longest T_1 corresponds to the H signal of DMF determining a relaxation delay of about 50 s.

Table 1
T_1 relaxation times of caffeine and DMF protons.

Molecule	Signal	Longitudinal relaxation times (s)		
		In ultrafiltered saliva	In $CDCl_3$	In D_2O
Caffeine	(CH_3)**10**	3.1	2.9	
	(CH_3)**11**	3.1	2.8	
	(CH_3)**12**	2.5	2.7	
	H**8**	5.8	3.2	
DMF	(CH_3)			8.7 and 5.0
	H			10.3

To calculate the analyte concentration, a scale factor of 0.108 was considered, corresponding to the ratio between the volume of the coaxial insert (V_c) and the volume of the NMR tube (V_t) [21,23]. The absolute concentration of caffeine (C_a) was determined by integration of the area of the methyl protons or of the H8 proton (I_a) and that of the methyl protons or of the aldehyde proton of DMF (I_s), using the relationship $C_a(\text{mol/L}) = (I_a/I_s)(V_c/V_t)C_s$, where C_s is the concentration of the external standard.

Peak identification was confirmed by spiking the sample with pure analyte.

2.7. Method

The proposed method was verified in terms of instrumental linearity, analytical recovery, and limits of detection and quantitation.

2.7.1. Instrumental linearity

Five blank saliva extracts in chloroform were spiked using five standard caffeine solutions in the concentration range 0.9–60 μg mL^{-1}, and were then analyzed. The relationship of the absolute integrals of analyte signals *versus* the corresponding concentration was tested by regression analysis.

2.7.2. Analytical recovery and repeatability

Blank saliva was spiked before sample preparation at three different concentration levels of caffeine (1.71, 4.25 and 8.40 μg mL^{-1}), each performed in triplicates. Samples were then treated and analyzed with the proposed procedure. The recovery was calculated as the ratio between the calculated concentration and the nominal concentration. Repeatability of the entire analytical procedure was tested in terms of relative standard deviation, RSD.

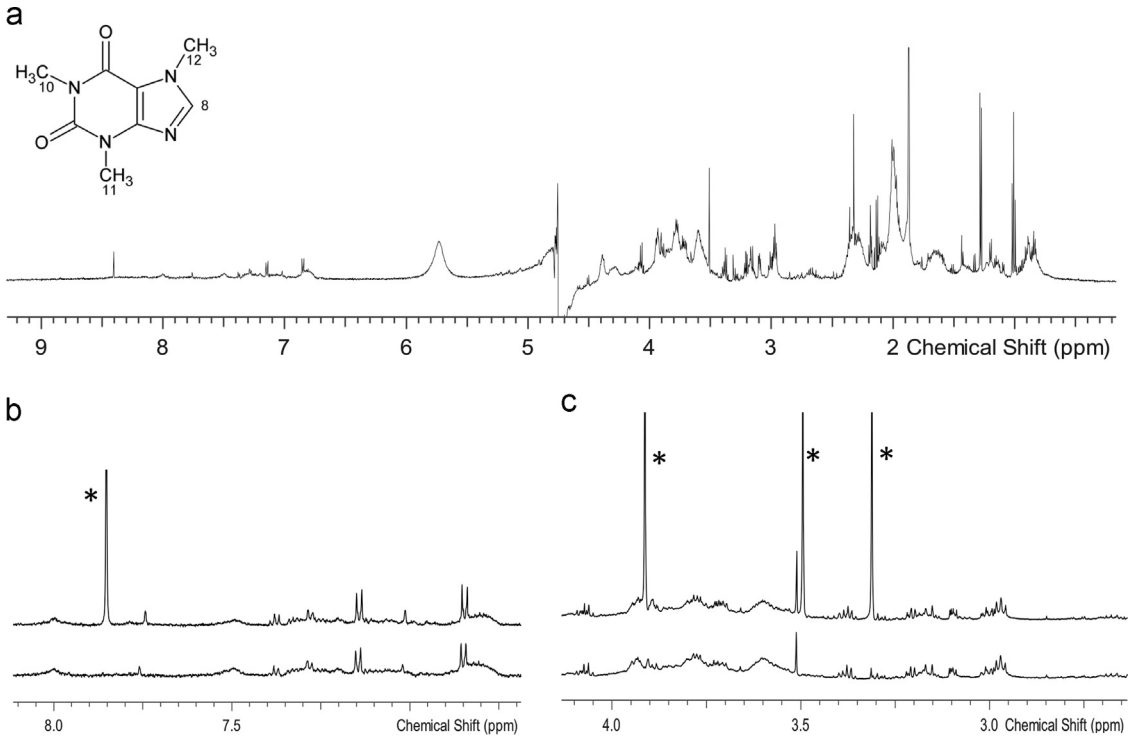

Fig. 1. Spectrum of a centrifuged saliva sample obtained after a 48 h abstinence from caffeine (a). Expanded aromatic (b) and aliphatic (c) regions of the same spectrum (bottom) and after spiking with caffeine (0.5 mM – top). Caffeine signals are marked.

2.7.3. Limits of detection and quantitation

The limits were determined in a blank saliva sample spiked with caffeine in the concentration range between 0.90 and 60 μg mL^{-1}. To calculate the LoD and LoQ, a signal to noise ratio of 3 and 10 was considered, respectively.

3. Results and discussion

3.1. Spectral analysis

In Fig. 1, a representative one pulse sequence spectrum of centrifuged saliva is shown.

The methyl signals of caffeine overlap with other signals in the aliphatic region while in the aromatic region, the H8 proton signal at 7.78 ppm is isolated. Unfortunately, the aromatic signal arises from only one proton, so the corresponding LoD and LoQ are higher than those reached considering the methyl signals of caffeine. A ^{1}H-CPMG experiment was also acquired with the aim to simplify the spectrum, filtering out signals from high molecular weight molecules which severely alter the baseline in the regular ^{1}H NMR spectrum but no significant improvement was observed (data not shown).

Therefore, the ultrafiltration step, as already reported in the literature for other biofluids [24], is necessary to quantify caffeine at the concentrations present in the aqueous samples during experimentations.

In Fig. 2a, a portion of the spectrum obtained after the ultrafiltration step is reported. Comparing it to the spectrum in Fig. 2b of the same sample spiked with caffeine, we can conclude that in this case, it is possible to integrate not only the H8 signal but also the methyl signal at 3.89 ppm.

In the case of the chloroform extracts, all the signals of interest resonate in areas of the spectrum clear of other signals; moreover, it is possible to use a higher receiver gain (because of the lower intensity of the water signal), and the analyte concentration is 4 times that in aqueous solution. The spectra of some extracts of one experimentation are reported in Fig. 3.

3.2. Sensitivity

The LoD and the LoQ were determined with 256 scans, corresponding to an acquisition time of 3 h 20 min. The results obtained considering the $-CH_3$ signals or the H8 proton signal of caffeine are reported in Table 2 for both the chloroform extracts and in the ultrafiltered saliva samples.

When the methyl signals are used, it is possible to quantify caffeine in saliva during a 24 h experimentation as the LoD and LoQ values are lower than the concentration found in real samples. The limits obtained for the H8 proton signal are lower than the caffeine concentrations found during experimentations only in the chloroform extract. In conclusion, with the chloroform extracts it is possible to integrate all the caffeine signals while in the ultrafiltered saliva, the needed sensitivity is reached only with the methyl signals with these experimental conditions.

3.3. Analytical results

To evaluate the accuracy and the extraction efficiency of the proposed method, recovery experiments were performed on blank saliva

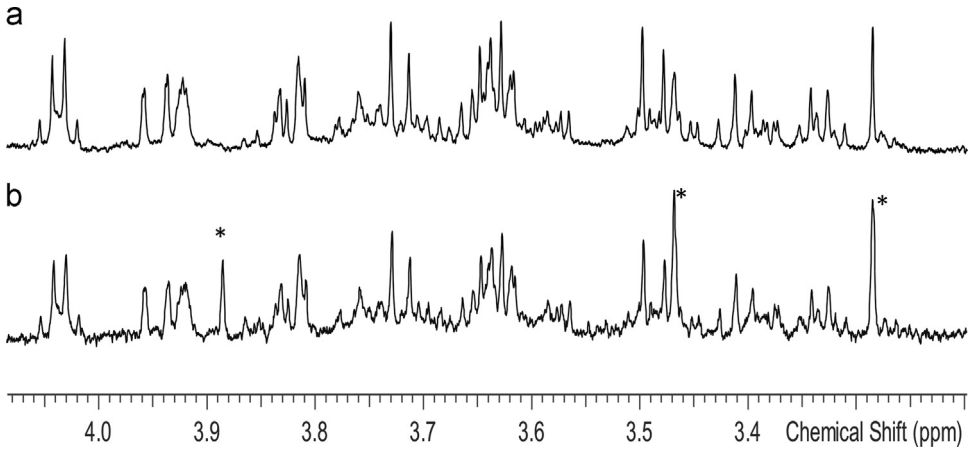

Fig. 2. Zoom of the aliphatic region of the spectra of ultrafiltered saliva (a) blank sample and (b) spiked with caffeine (48 μM).

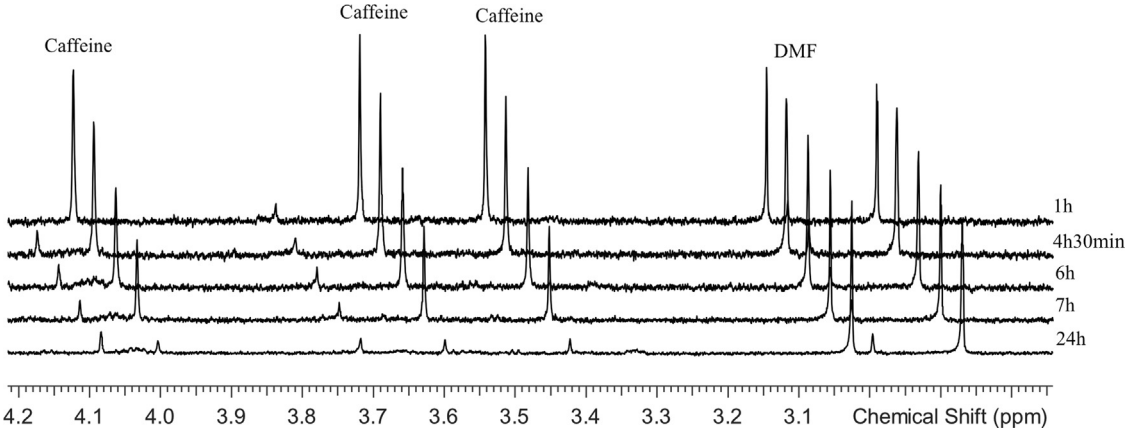

Fig. 3. Zoom of the aliphatic region of the spectra of saliva extracts at different times after caffeine intake.

samples, spiked at three different concentrations of standard caffeine both in chloroform extracts and in ultrafiltered samples (Table 3). In either case, the recovery is higher than 86%. In the extracts, the recovery is $>95\%$ at all spiking levels. The RSD values, ranging from about 4% at low caffeine concentrations to about 3% at higher levels, demonstrate the precision of the method.

Results in ultrafiltered samples are affected by a significant error due to the influence of signals interfering with the integration of the methyl signals in aqueous samples; this influence grows as the caffeine concentration becomes lower. In conclusion, to make a correct evaluation in ultrafiltered saliva, it is necessary to follow both the methyl signals and the H8 proton. Unfortunately, to do this, different experimental conditions are necessary: at least twice the number of scans needs to be acquired, with a longer relaxation delay. This would result in 7–8 h of acquisition time per experimental point. For this reason, we decided to follow the experimentation by analyzing only the saliva extracts in chloroform.

3.4. Instrumental linearity

To check the instrumental linearity, the concentration values obtained from standard caffeine chloroform solutions were plotted against the corresponding absolute integral of the analyte signal. Good linearity was achieved with a regression equation of $y=5.93\cdot10^{7}x+1.47\cdot10^{7}$ and a correlation coefficient $R^2=0.99949$. The linear relationship between the analyte concentration and instrumental response was thus confirmed for the concentration range 0.9–60 μg mL^{-1}, corresponding to 0.2–15 μg mL^{-1} in the matrix.

3.5. Application of the method

Data reported in Fig. 4 are normalized for the quantity of caffeine ingested. The caffeine quantity in the capsule is of 100 mg as declared by the producer, while the quantity of caffeine in the coffee beverage was determined by NMR spectroscopy of the beverage directly and of its chloroform extract.

Table 2
LoQ and LoD in the chloroform extracts and in ultrafiltered saliva, considering the methyl signals or the H8 proton signal of caffeine. The concentrations reported are the quantity of caffeine originally present in saliva; in the case of the chloroform extract, these concentrations are four times lower than those in the NMR sample.

	Caffeine signals	LoQ ($S/N=10$)		LoD ($S/N=3$)	
		mol L^{-1}	μg mL^{-1}	mol L^{-1}	μg mL^{-1}
Extract	CH_3[a]	$6.77\cdot10^{-7}$	0.13	$1.50\cdot10^{-7}$	0.03
	H	$3.40\cdot10^{-6}$	0.66	$7.57\cdot10^{-7}$	0.15
Ultrafiltered	CH_3 (3.89 ppm)	$3.51\cdot10^{-6}$	0.68	$7.80\cdot10^{-7}$	0.15
	H	$1.91\cdot10^{-5}$	3.71	$4.24\cdot10^{-6}$	0.82

[a] Average of the three signals.

The trends found are similar to the results already reported in the literature using HPLC [7], indicating that NMR is a suitable technique for CYP1A2 phenotyping. Compared to that method, our approach entails comparable sample preparation complexity and measuring time. Although the LoD and LoQ are slightly higher in our case, they are still lower than the caffeine concentration 24 h after administration, allowing a complete study of its clearance. The real advantage that we attach to the NMR determination is the much higher recovery,

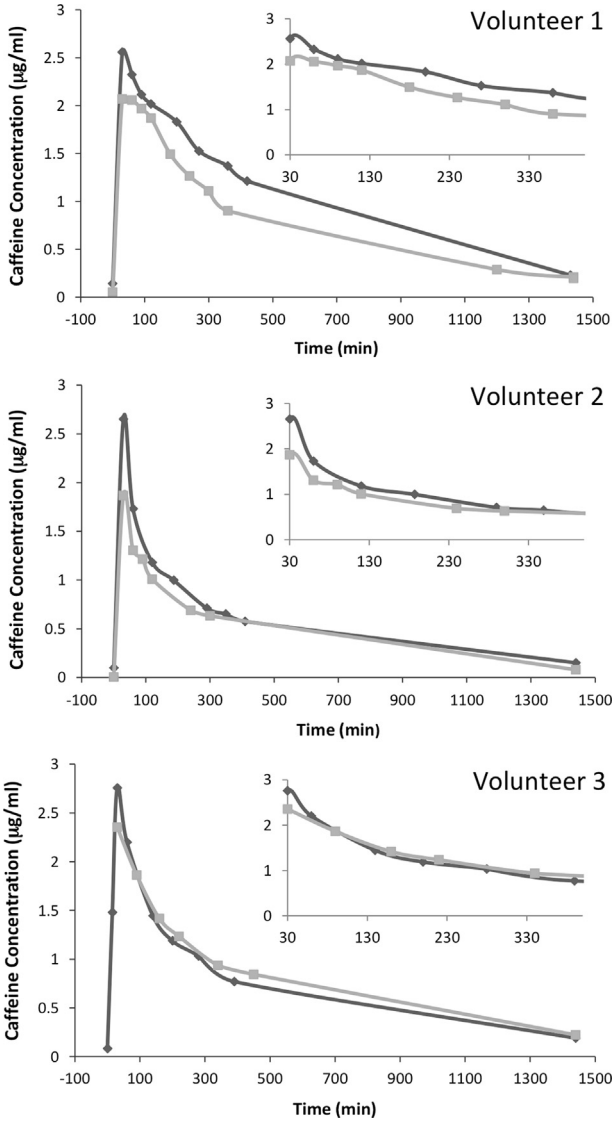

Fig. 4. Experimentation (black diamond=capsule, gray squares=espresso coffee).

Table 3
Recovery of caffeine in spiked saliva samples.

	Nominal caffeine μg mL^{-1}		Measured caffeine μg mL^{-1}		Recovery %	RSD %	Bias %
Extract	1.71	(±0.07)	1.64	(±0.04)	95.83	3.79	−4.17
	4.25	(±0.16)	4.13	(±0.02)	97.16	2.99	−2.84
	8.42	(±0.32)	8.21	(±0.015)	97.60	2.75	−2.40
Ultrafiltered	1.71	(±0.07)	2.01	(±0.34)	117.6	29.9	17.6
	4.25	(±0.16)	4.24	(±0.32)	99.9	14.9	−0.15
	8.42	(±0.32)	7.3	(±0.25)	86.7	6.8	−13.3

which translates in increased reliability. Moreover, the method we propose has better precision and accuracy than the HPLC method.

Another positive feature of the NMR approach is the intrinsic linearity of the technique and, in this particular case, the independence of the measurement from any interference or matrix composition.

4. Conclusions

In this work, we proposed an alternative method for the CYP1A2 phenotyping. The method proved to be fast, precise, and accurate. The sample preparation is less laborious than for the HPLC method. We present the first data obtained from the experimentation conducted on three volunteers. The maximum concentration was found at 30 min after caffeine intake, both as a capsule and as an espresso coffee beverage. The C_{max} ranged from 2.7 to1.9 $\mu g\,mL^{-1}$, or from $1.4\cdot10^{-5}$ to $9.6\cdot10^{-6}$ mol L^{-1}.

Of the several methods available for caffeine quantification in biofluids, the ones that employ saliva have the distinct advantage of a non-invasive sampling, particularly useful in the case of children. Compared to chromatographic methods, especially those that use MS for detection, the sensitivity of NMR is definitely lower. The same consideration holds if we compare NMR to a recently developed ELISA method [25]. Nevertheless, this is no concern, as the amounts of caffeine in saliva are sufficiently high when it is given as drug, making the NMR method adequate for CYP1A2 phenotyping.

Although the method we propose does not allow to determine caffeine directly in saliva, the direct extraction in deuterated solvent permits very low concentrations to be detected. Besides, the LoD and LoQ depend on the choice of a convenient experimental time and they can be considerably improved.

One factor that could be improved is the reference standard. We chose DMF because we wanted to be able to quantify both the methyl protons and H8 in caffeine. DMF has the peculiar feature to have signals both in the aromatic and in the methyl regions, although the T_1 relaxation times of all its protons are much longer than those of caffeine, increasing the necessary acquisition time. Our study showed that quantification is achieved conveniently only in the methyl region, opening the way for the search of more specific standard compounds, *i.e.*, with signals only in that region and with shorter relaxation times.

Unfortunately, we were not able to observe paraxantine signals to allow its quantification. The paraxantine/caffeine ratio in saliva, 4 h after administration of caffeine has been shown to be an accurate measurement of CYP1A2 activity and it would be interesting to determine *via* NMR. Attempts in this direction are being pursued.

Acknowledgment

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