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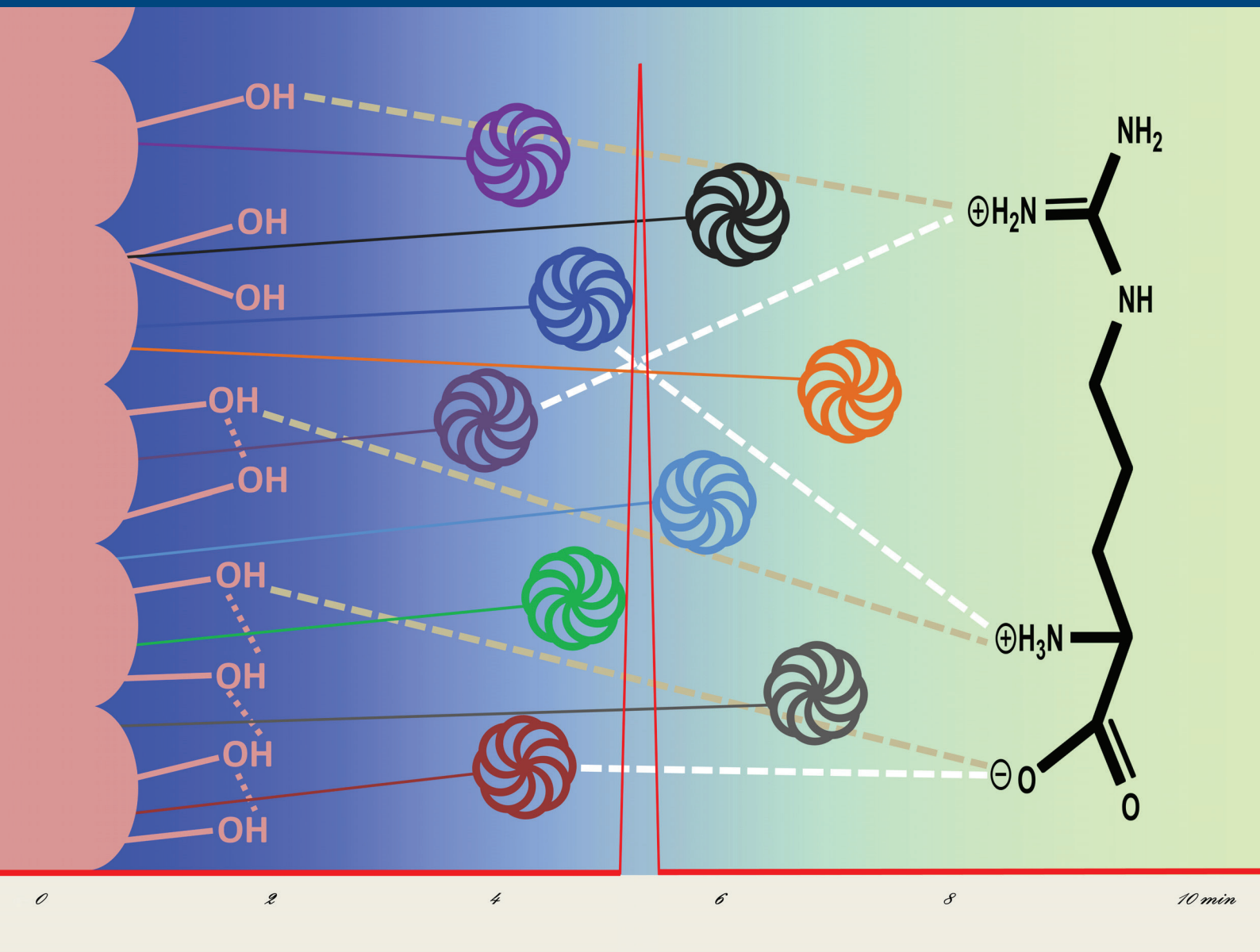

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RESEARCH ARTICLE

Supercritical fluid extraction–supercritical fluid chromatography of saliva: Single-quadrupole mass spectrometry monitoring of caffeine for gastric emptying studies†

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Saliva is an attractive sampling matrix for measuring various endogenous and exogeneous substances but requires sample treatment prior to chromatographic analysis. Exploiting supercritical CO_2 for both extraction and chromatography simplifies sample preparation, reduces organic solvent consumption, and minimizes exposure to potentially infectious samples, but has not yet been applied to oral fluid. Here, we demonstrate the feasibility and benefits of online supercritical fluid extraction coupled to supercritical fluid chromatography and single-quadrupole mass spectrometry for monitoring the model salivary tracer caffeine. A comparison of ^{13}C- and ^{32}S-labeled internal standards with external standard calibration confirmed the superiority of stable isotope-labeled caffeine over nonanalogous internal standards. As proof of concept, the validated method was applied to saliva from a magnetic resonance imaging study of gastric emptying. After administration of 35 mg caffeine via ice capsule, salivary levels correlated with magnetic resonance imaging data, corroborating caffeine's usefulness as tracer of gastric emptying ($R^2 = 0.945$). In contrast to off-line methods, online quantification required only minute amounts of organic solvents and a single manual operation prior to online bioanalysis of saliva, thus demonstrating

Article Related Abbreviations: BPR, backpressure regulator; LAI, laboratory-acquired infection; LLOQ, lower limit of quantification; MRI, magnetic resonance imaging; QC, quality control; SFC, supercritical fluid chromatography; SFE, supercritical fluid extraction; SIL-IS, stable isotope-labeled internal standard; TDM, therapeutic drug monitoring

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the usefulness of CO_2-based extraction and separation techniques for potentially infective biomatrices.

KEYWORDS
carbon dioxide–based separation, online sample preparation, saliva, supercritical fluid chromatography, supercritical fluid extraction

1 | INTRODUCTION

Biological matrices often require several treatment steps prior to chromatographic quantification of target analytes. Indeed, typical sample preparation techniques such as LLE or SPE constitute the most labor-intensive and error-prone stage of bioanalysis, second only to sample generation itself (Figure 1A and Supporting Information S1) [1]. Moreover, each additional procedure associated with sampling and sample preparation exposes operators to biological (infectious samples) and toxicological (organic solvents) risks, as well as aggravating the environmental impact [2].

Comparing medically relevant biomatrices and the sample treatment they require, oral fluids alleviate some of these concerns due to their easy availability and aqueous composition, enabling low-effort sample preparation such as protein precipitation or even dilute-and-shoot approaches [3]. Since the salivary concentration of many compounds correlates with the nonprotein-bound (i.e., active) plasma fraction that is responsible for biological effects [4], oral fluid is gaining popularity as a continuously updated, short-term memory device for therapeutic drug monitoring (TDM) [5], toxins [6], pathogens [7,8], neoplasms [9], hormonal status [10], inflammation [11], metabolic dysregulation [12], neurodegenerative processes [13], and phenotyping/genotyping [14].

In terms of safety, saliva generally poses a lower risk of infection, both for sampling staff—due to its noninvasive nature and advances in sampling devices [15]—and for operators, since sample infectiousness is comparatively low [16]. However, as demonstrated by COVID-19 where saliva is valued for its diagnostic utility [17] but known for its infectiousness [18], oral fluids too place health care and life science professionals at risk. Indeed, laboratory-acquired infections (LAIs) via saliva have been documented for viral (e.g., hepatitis B and herpes B) [19] and discussed for bacterial diseases (e.g., tuberculosis) [7,20] (Supporting Information S2). In the absence of a centralized registry for LAIs, the unrecorded number of infections is bound to be higher. Hence, the choice of bioanalytical methodology should reflect the risks posed to life science professionals—especially in light of the current pandemic [21,22].

CO_2-based techniques such as supercritical fluid extraction (SFE) and supercritical fluid chromatography (SFC) attenuate chemical toxicity and environmental pollution by promoting the usage of CO_2 as a safe, affordable, and green alternative to organic solvents [23] while maintaining analyte stability [24]. Combination of SFE with SFC also mitigates the risk of infection: Although supercritical CO_2 does not reliably lead to sterilization due to how fast separation is achieved [25], seamless interfacing of extraction and analysis reduces the number of manual handling operations and thereby operators' exposure to solvents and samples (Figure 1B) [26]. Thus, online coupling of CO_2-based techniques reduces hardware requirements (joint

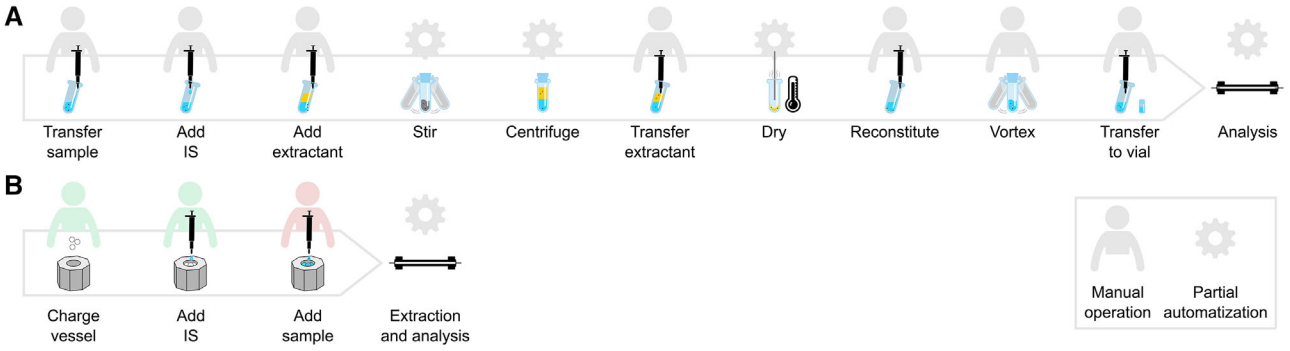

FIGURE 1 Sample treatment protocols: (A) LLE. (B) Supercritical fluid extraction and chromatography. See Supporting Information S1 for additional treatment procedures

usage of CO_2-pumps, etc.), lowers the economic and environmental cost, and increases speed and safety of bioanalytical separations [27].

Quantitative determinations by online SFE–SFC have been realized for blood [28], plasma [29], serum [30], urine [31], plant-based [32], and nonbiological matrices [33], largely made possible by the development of sensitive MS interfaces [34]. To the best of our knowledge, SFE–SFC-MS of saliva has not been reported [23,27,35–38].

To investigate the applicability of SFE–SFC to oral fluids, a widely applicable target analyte was chosen: the salivary tracer caffeine (1,3,7-trimethylxanthine). Although flat, caffeine is still a very drug like, moderately lipophilic (experimental log*P* −0.07 [39]) molecule that passes biological membranes/barriers. It shows zero violations of Lipinski's rule of five 5s and high permeability (Caco-2). At the same time, caffeine is well soluble in water (21.7 g/L) at room temperature. In the form of caffeine citrate, it is used therapeutically as respiratory stimulant in preterm neonates and has been studied under TDM [40,41]. As tool compound with benign physicochemical properties, it has been used in CYP1A2 phenotyping [42–45], drug transit/delivery studies [46,47], and gastrointestinal tract (GIT) transit experiments [48–50]. For our study, detection of caffeine was performed by single-quadrupole MS to achieve the sensitivity required for low-dose biomarker studies without incurring the economic strain of other MS techniques (e.g., triple-quadrupole MS/MS). The aim of this study was to develop and optimize an SFE–SFC–MS method capable of quantifying salivary concentrations after low-dose administration of caffeine. The applicability of the novel method was tested on a healthy volunteer in a magnetic resonance imaging (MRI)-validated gastric emptying study, examining the correlation of salivary caffeine levels with gastric volume.

2 | MATERIALS AND METHODS

2.1 | Chemicals

CO_2 (99.995% purity) was obtained from Air Liquide (Duesseldorf, Germany). Drying/nebulizing gas (N_2) for ESI-MS was generated online (cmc Instruments, Eschborn, Germany). Co-solvents (methanol and 2-propanol from Carl Roth, Karlsruhe, Germany) and additives (formic acid from Fisher Scientific, Geel, Belgium; 25% aqueous solution of ammonia from Sigma-Aldrich; Steinheim, Germany) were obtained in LC-MS grade. The adsorbent diatomaceous earth (ISOLUTE HM-N) was purchased from Biotage Europe (Uppsala, Sweden). Sigma-Aldrich also provided reference standards, with the exception of ^{32}S-6-thiocaffeine, which was synthesized in-house according to Rico-Gómez et al. [51,52].

2.2 | Instrumentation

Extraction and chromatography were performed on a Nexera SFE-SFC/UHPLC switching system (Shimadzu Corporation, Kyoto, Japan) consisting of one LC-30ADSF pump (liquid CO_2), two LC-20ADXR pumps with DGU-20A5R degassers (modifier/makeup), two SFC-30A backpressure regulators (BPRs), a SIL-30AC autosampler (for SFC), an SFE-30A extraction unit (for SFE–SFC), a CTO-20AC column oven, and a CBM-20A communication module (Figure 2A). Analytes were detected via single-quadruple MS (LCMS-2020) equipped with an ESI source, except for uric acid, which was detected via photodiode array (SPD-M20A). The system was controlled by LabSolution (Version 5.82).

The stationary phases Luna-NH_2 (150 × 3.0 mm, 5 μm), Lux Amylose-2 (150 × 4.6 mm, 5 μm), Lux i-Amylose-3 (150 × 2.0 mm, 3 μm), Lux i-Cellulose-5 (100 × 3.0 mm, 3 μm), and Synergi Polar-RP (150 × 4.6 mm, 5 μm) were purchased from Phenomenex (Aschaffenburg, Germany); Torus Diol (100 × 2.1 mm, 1.7 μm) and XTerra MS C18 (100 × 2.1 mm, 3.5 μm) from Waters (Milford, USA); and Gemini C18 (100 × 4.6 mm, 5 μm) from VWR (Karlsruhe, Germany).

MRI was performed in a 1.5 Tesla Siemens Magnetom Aera (Siemens Healthcare GmbH, Germany) and analyzed manually by Horos v2.2.0 software (The Horos Project).

2.3 | Standard solutions

Aliquots of stock solutions (100 μg/mL) of the external standard caffeine and the internal standards (IS) $^{13}C_3$-caffeine (1,3,7-tri-(^{13}C-methyl)xanthine) and ^{32}S-6-thiocaffeine were prepared weekly in Milli-Q water and stored at −20°C. Working solutions of the external standard (10 and 1 μg/mL) and the IS (5 μg/mL) were prepared daily and used to spike blank saliva obtained from healthy individuals abstaining from caffeine-containing products.

2.4 | Sample preparation

Study samples were collected in SafeSeal microtubes (Sarstedt, Nümbrecht, Germany) and stored at −80°C until analysis. SFE-extraction vessels (0.2 mL inner volume) were 50% (*v*/*v*) filled with adsorbent (100 μL bulk volume) and spiked with 10 μL of IS stock solution (50 ng). Note that 100 μL of sample was introduced onto the adsorbent and loaded into the autosampler without further treatment.

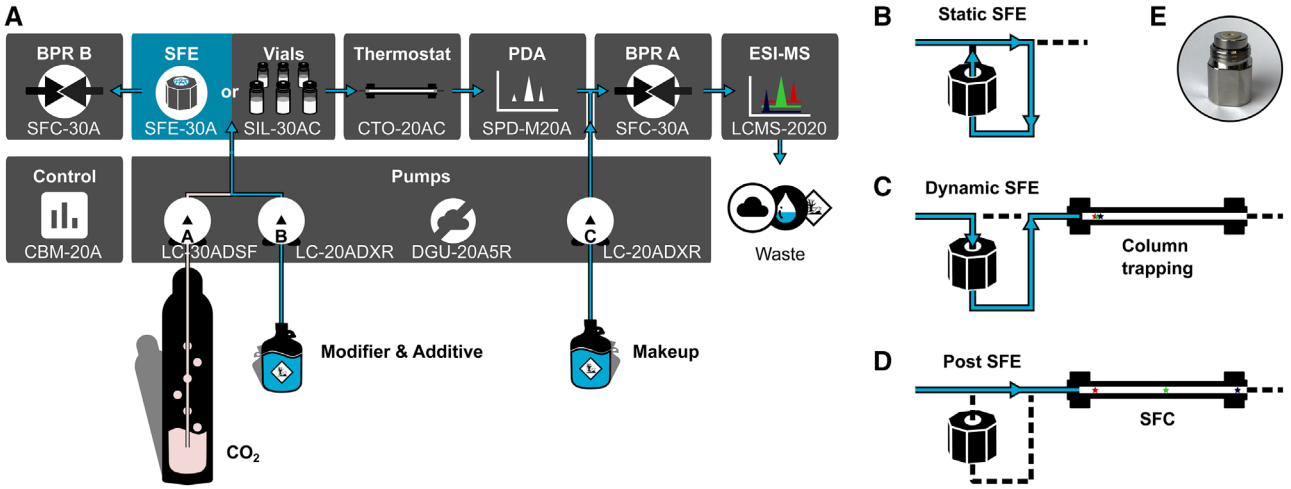

FIGURE 2 Instrumental setup. (A) Apparatus. (B–D) Flow diagram (modified from Wicker et al. [33]). (E) Extraction vessel

TABLE 1 Dynamic system settings

Mode	Start (min)	End (min)	CO_2 (%)	Methanol (%)	Flow (mL/min)	BPR B[a] (bar)
Static SFE	0.00	1.50	100	0	3	130
Dynamic SFE	1.51	3.49	100	0		
SFC	3.50	7.00	97	3		400
Wash	7.01	8.33	0	100	1	
Equilibration	8.34	10.00	100	0	3	130

[a]Pre-column backpressure regulator.

2.5 | Online extraction and chromatography

The postcolumn BPR A was set to 105 bar (50 °C). System settings are summarized in Table 1. During static SFE, pure CO_2 was directed into the extraction vessel (Figure 2B). Then, during dynamic SFE, the flow was directed through the vessel and onto the column, trapping analytes at the head of the column (Figure 2C). A Luna-NH_2 column was used for the final method. During SFC, flow was redirected to circumvent the extraction vessel, and 3% methanol (isocratic) was added to CO_2 to overcome trapping (Figure 2D). Finally, a wash step was included to clean the column from co-extracted matrix, prior to re-equilibration.

2.6 | Mass spectrometry

The final method employed ESI-MS detection in positive mode (interface voltage 4.5 kV) with nitrogen as nebulizing (1 L/min) and drying gas (20 L/min). Analyte precipitation due to CO_2 decompression was avoided by adding 0.05 mL/min of makeup (2-propanol containing 2% water and 0.75% formic acid to aid ionization). The needle wash solvent was methanol. Optimization yielded 500, 350, and 300°C for heat plate, interface, and desolvation line temperature, respectively. With the exception of uric acid, analytes were detected in SIM mode as protonated molecular ion $[M + H]^+$ (Supporting Information S3).

2.7 | Validation

Blank saliva was obtained from healthy volunteers abstaining from xanthine-containing foods (chocolate) and beverages (caffeinated drinks, cocoa) for at least 3 days prior to sampling. Calibrators were spiked to yield 31.25, 62.5, 125, 250, 500, and 1000 ng/mL caffeine, corresponding to absolute amounts of 3.125, 6.25, 12.5, 25, 50, and 100 ng, respectively, per sample (100 µL saliva). Three sets of calibrations were performed on different days to assess inter- and intrarun variability. Low, medium, and high quality control (QC) samples were spiked to yield 62.5, 500, and 1000 ng/mL to judge accuracy and precision according to acceptance criteria set by FDA [53] and EMA [54].

Accuracy is presented as the ratio of the determined value to the nominal value, and precision as closeness of individual measures, as defined by EMA [54]. Three (^{32}S-6-thiocaffeine) and six ($^{13}C_3$-labeled caffeine and external standard calibration) replications were performed in accordance with tiered [55] and decision-based validation [56]. Selectivity and carry-over were determined by comparing signals obtained from blank samples to that of the lower LOQ (LLOQ; $n = 6$) as defined by EMA [54]. Benchtop-, autosampler-, and long-term stability have been demonstrated elsewhere [50]. Clinical results were confirmed by a published reference method (fully validated RP-HPLC-MS/MS) [48].

2.8 | Application

The method was applied to one healthy volunteer of a study conducted in compliance with the Declaration of Helsinki (2013, Fortaleza, Brazil) and the (Model) Professional Code for Physicians in Germany (amended 2015 in Frankfurt, Germany) after approval by the ethics committee of the University Medicine Greifswald (BB 071/17a). The volunteer gave written informed consent and was insured to cover risks arising from study participation and commuting accidents. EMA and FDA guidance for bioavailability and bioequivalence studies were observed [57,58]. The volunteer abstained from caffeine-containing products for at least 3 days prior to administration of 35 mg of caffeine with 20 mL of water. The dose of caffeine was administered as frozen solution inside a capsule of pure frozen water to avoid direct contamination of the oral cavity and thus saliva.

The ice shell and its filling were prepared as described by Sager et al. [48]. To form the outer shell, deionized water was frozen in a specific silicon mold. Subsequently, 0.5 mL pre-cooled solution with 35 mg caffeine (70 mg/mL) and 250 mg saccharine sodium (500 mg/mL) was filled in the prepared ice capsule underparts. Filled capsules were completely frozen again at −80°C and subsequently closed by adding a cap of 0.3 mL water, which immediately froze on the cold capsule body.

Saliva was sampled by the volunteer herself via direct transfer of 0.5–1 mL saliva into 2-mL-tubes without stimulation or the use of additional sampling devices. Sampling took place over the course of 1 h (every 2 min for the first 20 min, every 5 min for the following 20 min, and finally every 10 min).

Gastric emptying was monitored by MRI, with measurements taking place in head supine position 1 min before each saliva sampling, using strongly T2-weighted HASTE sequence with 1000 ms repetition time, 198 ms echo time, 5 mm slice thickness, and 1 mm interslice gap to achieve a voxel size of 12.2 mmş for visualization and calculation of gastric volumes. To reduce motion artifacts, high-resolution coronal sequences were acquired during single inspiration breath-hold. Measured caffeine concentrations were normalized on c_{max} of pharmacokinetic profile; gastric volume data were normalized on starting volume (resting volume plus administered volume) to correlate the relative amount of emptied fluid with relative amount of absorbed caffeine (Figure 3).

2.9 | Computational methods

All molecules and complexes were prepared with the Molecular Operating Environment (MOE) [48]. Initial three-dimensional structures were optimized using the AMBER force field with extended Hückel theory parameters. Hydrophilic contact preferences were estimated using the surface builder tool in MOE. For quantum chemical calculations, geometries and harmonic frequencies were calculated with Gaussian 09 [59] using the B3LYP hybrid functional with 6–311++G** basis set. Electronic energies were obtained from single point energy calculations at the MP2 level of theory. The gas phase proton affinity was estimated according to the formula proton affinity (PA) = $-\Delta E_{elec} - \Delta ZPE + 5/2\,RT$.

3 | RESULTS AND DISCUSSION

Since this was the first salivary application of bioanalytical SFE–SFC–MS, method development and optimization concerned extraction (selectivity and completeness), chromatography (greenness and time), and MS detection (sensitivity and reproducibility).

3.1 | Supercritical fluid extraction

Saliva presents analytical chemists with an easily collectible, hypotonic biomatrix consisting mainly of water (99%) and electrolytes (Supporting Information S2) [60]. Direct injection is not advisable due to the presence of glycoproteins (mucins, enzymes, antimicrobial factors), a wide range of low molecular weight biomolecules (peptides, amino acids, carbohydrates, urea, hormones and their precursors, nucleotides), including substances related to caffeine (xanthine, hypoxanthine, and uric acid) [61,62], a small lipophilic fraction (mostly fatty acids) [63], cells (human leukocytes, desquamated epithelial cells, microbial colonizers), and fragments thereof [64].

Previously described HPLC methods used protein precipitation to prevent clogging of the chromatographic sys-

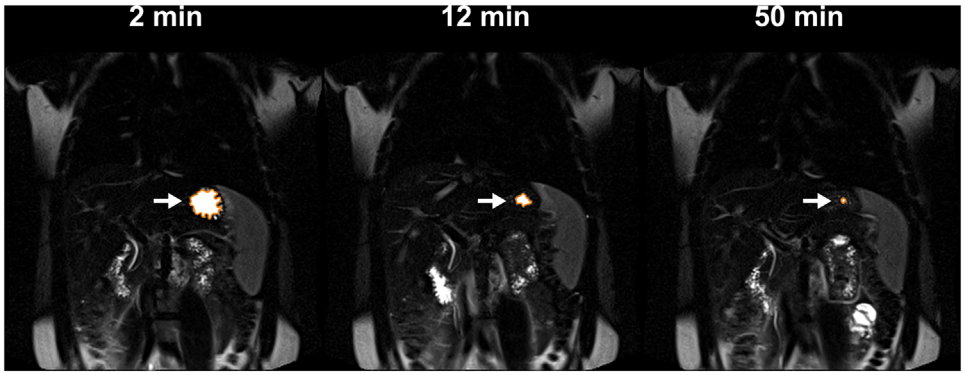

FIGURE 3 Coronal magnetic resonance image of the abdomen after oral administration of a caffeine containing ice-capsule and 20 mL of water. Gastric content volumes were tracked manually (arrow) and calculated by two different observers (acceptable range of 10% for volumes above 10 mL)

tem [48], in combination with RP-conditions to favor early elution of polar interferences such as alkali metal clusters and metabolites of caffeine [65,66]. Switching to SFE–SFC–MS, it was hypothesized that extraction by heptane-like CO_2 would preferentially extract low-polarity analytes, NP-conditions causing the target analyte to elute prior to co-extracted ions and metabolites.

The literature is rich in reports concerning the solubility of caffeine in supercritical CO_2 [67–72], including the original proof-of-concept for online (nonquantitative) SFE–SFC by Sugiyama et al. [73]. When working with solid matrices (e.g., coffee beans) and high analyte concentrations such as 0.7–1.6% (*m*/*m*) in the case of *Coffea arabica* [74], recovery correlates positively with residual moisture (ranging from 0 to 20%), pressure (100–200 bar), and total extraction time (up to hours). Less information was available on SFC of low-dose biological samples, especially biological fluids. In the case of salivary samples, quantification via a published HPLC method revealed target salivary caffeine c_{max} after single dose administration of 35 mg caffeine to be well below 1 μg/mL (i.e., 1 ppm (*m*/*m*)).

Initial SFE experiments identified the adsorbent as the dominant extraction parameter in SPE [31] or supported liquid extraction [75] by SFE. Although extraction without any adsorbent yielded the highest recovery, the possibility of leakage from the extraction chamber rendered handling of higher sample volumes inconvenient. Employing too strong of an adsorbent, on the other hand, resulted in almost no caffeine being recovered (*viz.* activated carbon). Cellulose-based adsorbents such as cotton swabs and filter paper yielded intermediate recovery (cotton pad) but also analytical artifacts and peak splitting. Diatomaceous earth (SiO_2) provided excellent recovery, peak shapes, and the lowest level of noise (Figure 4).

Unlike with highly concentrated caffeine samples, there was no observable gain in recovery beyond 1.5 min static and 2.0 min dynamic extraction time when applied to

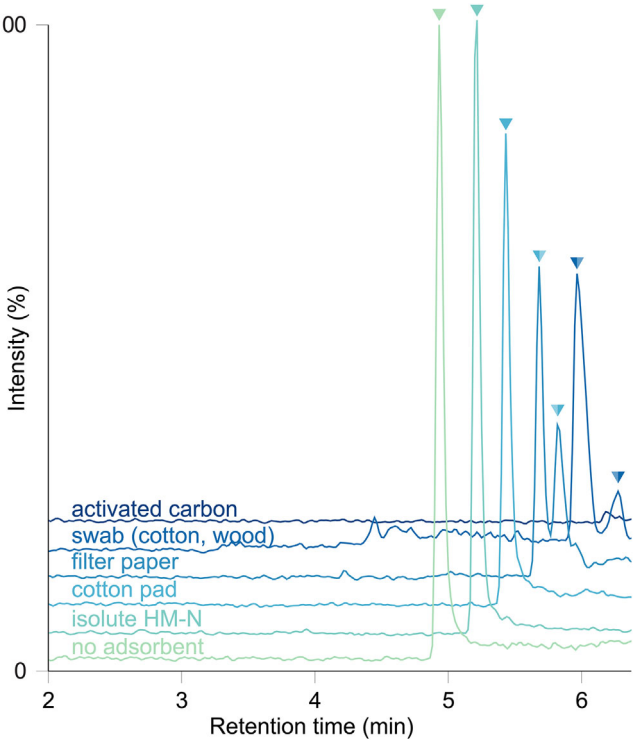

FIGURE 4 Effect of the adsorbent on peak shape and recovery of 50 ng caffeine from saliva

1 ppm caffeine solutions. Furthermore, no difference was observed at within the investigated temperature (25−75 °C) and pressure range (BPR A 100−150 bar) at this concentration. Interestingly, peak areas were not affected when comparing dried and wet samples, which allowed the streamlining of sample preparation compared to previous SFE-SFC methods that required 1 h of drying [76]. However, when working with nondried samples, matrix volume was shown to play a vital role in determining robustness: When extracting samples containing >150 μL liquid, depressurization during the switch from static to dynamic extraction

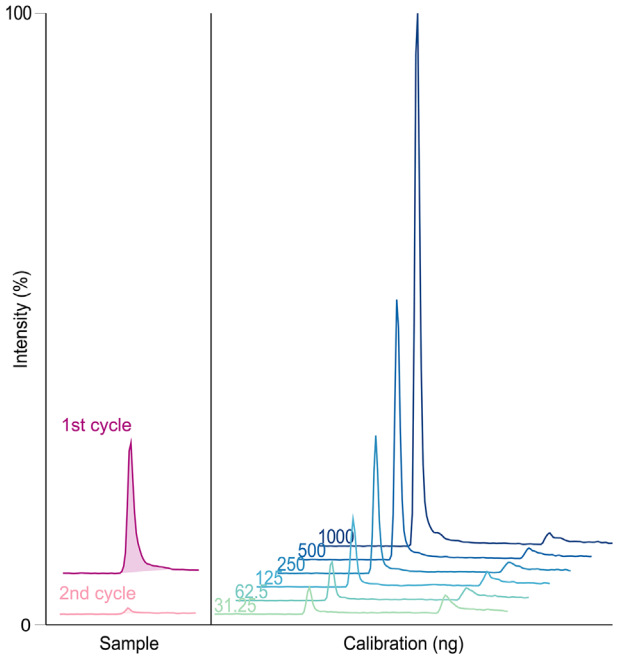

FIGURE 5 Recovery after repeated extraction of caffeine from saliva. The first extraction cycle yielded quantifiable results; subsequent extraction yielded signals below LLOQ

risked matrix being pushed out of the extraction vessel, leading to carry-over into the following analysis. Although saliva is more viscous than pure water, up to 100 µL of matrix could be supported by adsorbent in 0.2 mL extraction vessels before breaching vessel integrity during valve switching. As noted by Abrahamsson et al. [75], the loading capacity could be increased by decelerating vessel pressurization and by drying, although this was not necessary here to attain the required sensitivity (target range: 31.25–1000 ng/mL).

Repeated extraction of the same sample (before and after drying) indicated near-completeness during the first cycle (Figure 5). Due to the hydrophilic nature of possibly interfering compounds in the biomatrix and the lipophilicity of the extractant CO_2, a pre-column split was not necessary (BPR B was set to 130–400 bar). For details concerning the use of split-flow systems by differential pressure control between two BPRs, see the work published by Sakai et al. [77,78].

3.2 | Supercritical fluid chromatography

The stationary phase serves two functions in online SFE–SFC: (1) focusing the target analyte at the column head during the dynamic stage of extraction and (2) separating analytes from interferences during the chromatographic stage. Our aim was to avoid the need for two separate columns (i.e., finding an analytical column that also could function as a trapping column) while at the same time separating the target analyte in as little time and at as low a modifier concentration as possible (throughput, greenness).

Due to its use as test substance for stationary phase characterization, a review of the literature paints an unusually clear picture of what elution behavior to expect from caffeine [79–81]. Using methanol as modifier, Upnmoor and Brunner observed the highest retention of caffeine on unmodified silica, followed by C18-/C8-, NH_2- and CN-modified stationary phases [82]. Thus, classical RP (C18) [73] and NP (unmodified silica) [83] stationary materials would require excessive amounts of co-solvent to speed up analysis.

An exploratory column screening comprising C18-, phenyl ether-, polysaccharide-based, diol-, and NH_2-modified silica suggested the latter two HILIC materials to yield short and efficient run times (Supporting Information R1). However, the diol phase was not suitable for analyte-trapping, and hence performed well only in SFC mode (direct injection of standard solutions), but not SFE-SFC, as analytes eluted even when no modifier was added (Supporting Information R2). To prevent the resulting peak broadening would have required an additional trapping column. The aminopropyl-modified Luna-NH_2 on the other hand was effective at both trapping (dynamic extraction conditions: 100% CO_2) and releasing the target analyte (3% methanol, Supporting Information R3) and separate caffeine from its increasingly polar metabolites theophylline, theobromine, and paraxanthine (Figure 6).

Upnmoor and Brunner [82] and Berger [84] note the reversal of elution order of caffeine and its metabolites depending on mobile phase composition, suggesting divergent retention mechanisms in the presence/absence of polar modifiers. We, too, noticed orthogonal elution patterns seemingly defiant of simplistic NP- or RP-like elution behavior while screening for a suitable IS. Curious as to how to explain these observations, a xanthine test mixture was investigated experimentally and computationally for more information on the retention mechanisms of xanthine analogues in SFC.

3.3 | Predicted versus observed elution order

Aminopropyl-modified silica is compatible with NP, RP, and HILIC mode in LC (i.e., a hybrid stationary phase). When using only small amounts of the polar mobile phase constituent, HILIC stationary materials retain polar analytes longer than nonpolar analytes, which is consistent with NP-like behavior. This trend should extend to SFC when using heptane-like CO_2 modified by minor amounts

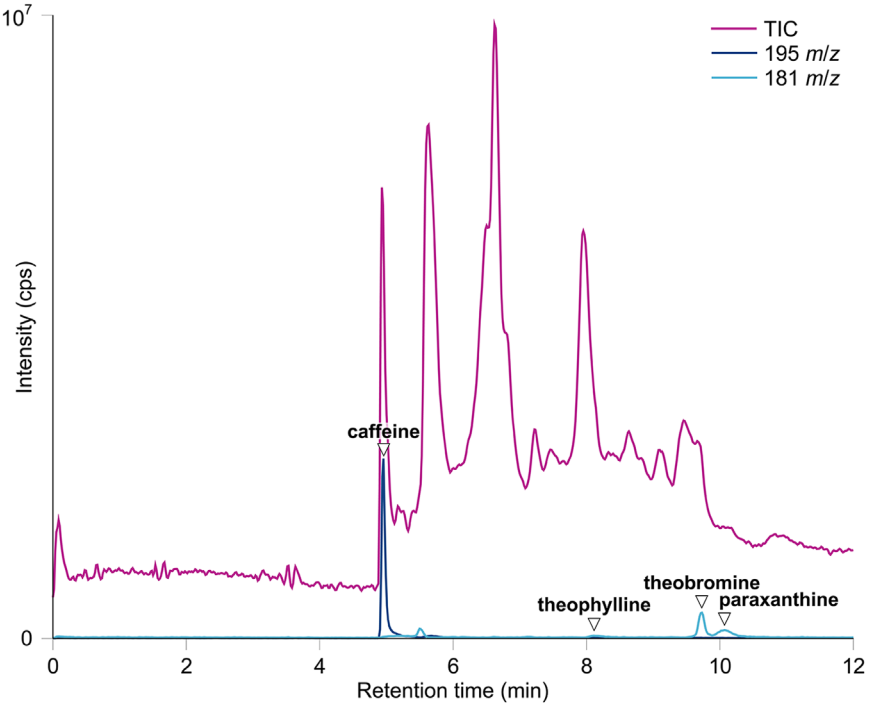

FIGURE 6 SFE-SFC chromatogram of salivary xanthines obtained from a healthy volunteer after consumption of one cup of coffee

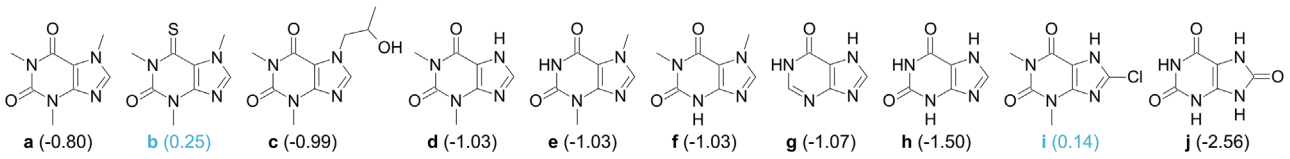

FIGURE 7 Xanthine analogues (clog*P* in brackets) in order of elution. Examples of seemingly orthogonal elution patterns in blue. a, caffeine; b, 6-thiocaffeine; c, β-hydroxypropyltheophylline; d, theophylline; e, theobromine; f, paraxanthine; g, hypoxanthine; h, xanthine; i, 8-chlorotheophylline; j, uric acid

of methanol [85]. Naively reasoning, elution order of xanthine derivatives should therefore be inversely correlated with log*P*, that is, elution of trimethylated < dimethylated < monomethylated < unmethylated analogues, followed by oxygenation products (Figure 7).

The observed order of elution indeed supported NP-like behavior at the macroscopic level but broke down at the level of specific analytes: 6-thiocaffeine, the most lipophilic of the studied analytes due to the lipophilic effects of the sulfur atom, eluted after caffeine; similarly, 8-chlorotheophylline, which contains a lipophilic halogen substituent and is a commonly employed IS for caffeine in RPLC-MS, was the penultimate analyte to elute [48]. The elution time discrepancy between it and caffeine disqualified this analyte as IS in SFC. Similarly, 7-(β-hydroxypropyl)theophylline, another IS of caffeine in RPLC, eluted significantly after caffeine but prior to theophylline, the additional hydrogen bonding site notwithstanding [86]. Apart from emphasizing the different IS requirements between RPLC and SFC, these findings raised the question of whether these deviations from NP-like behavior should be attributed to the orthogonality of HILIC (HILIC≠NP) or SFC (SFC≠NP).

As shown in Figure 8, neither is necessarily the case, since prediction of retention solely on the basis of clog*P* is an oversimplification, especially with a view to the only partly available experimentally determined log*P* values that differ slightly, for example, in the case of caffeine: clog*P* −0.80 versus log*P* −0.07. Taking the thio-analogue of caffeine as an example, quantum chemical calculations (DFT; B3LYP/6-311++G**) yielded a potential energy surface for S···H that was flatter than for O···H with similar binding energy, suggesting a wider deviation from the optimal hydrogen bond geometry and hence stronger interactions with the stationary phase (Figure 8a and b) [87–89]. In the case of 7-(β-hydroxypropyl)theophylline, the secondary hydroxy group was shown to engage in intramolecular hydrogen bonding that occupied possible interaction sites, exposing a larger hydrophobic area on the eastern part of the molecule that caused early elution (Figure 8c). Finally, comparing theophylline with its chloro-analogue, a halogen substituent should

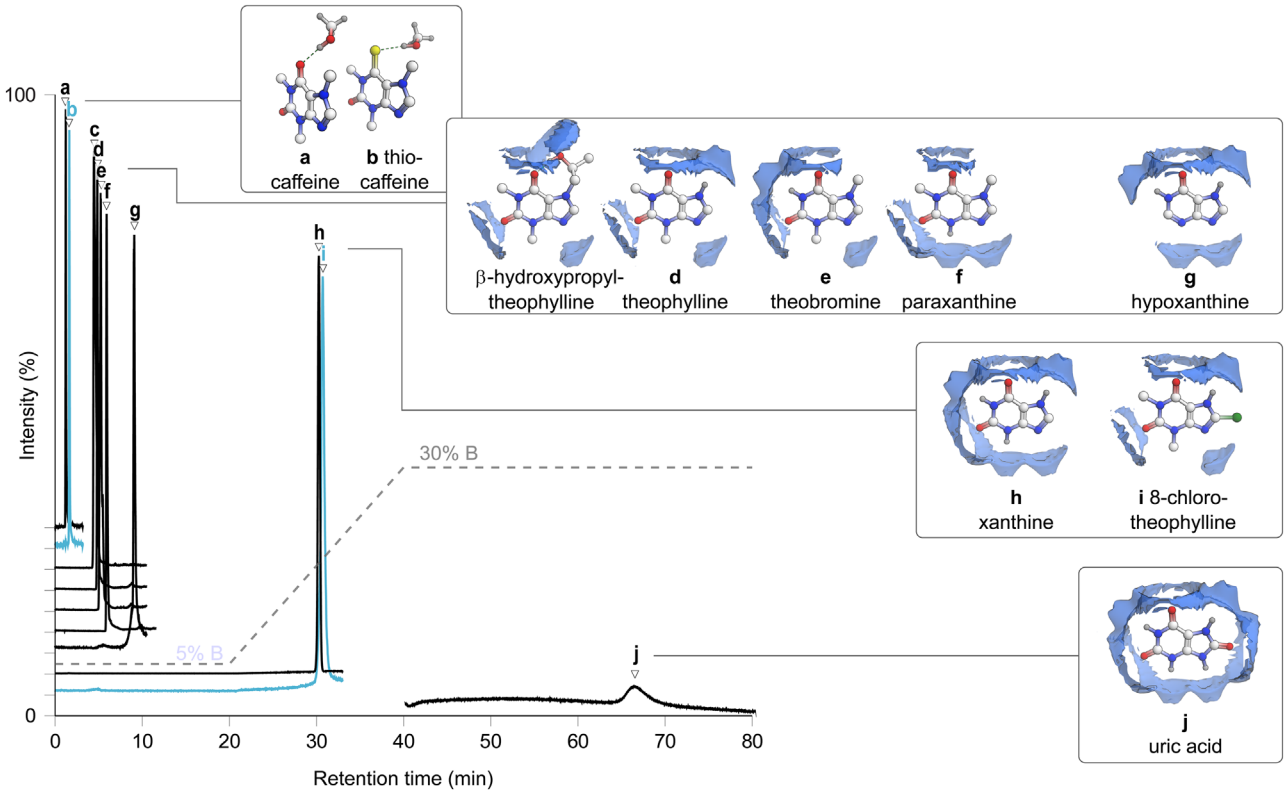

FIGURE 8 SFC chromatograms and surface geometries of xanthine analogues. Note the sharper hydrogen bonding angle for thiocaffeine versus caffeine (DFT; B3LYP/6-311++G**), intramolecular hydrogen bonding in β-hydroxypropyltheophylline, and the gradual increase in polar surface area (dark blue) in order of elution. Although 8-chlorotheophylline seems to deviate from this trend, late elution can be explained by ionic interactions due to increased acidity

enhance hydrophobicity and facilitate early elution. However, halogens also increase acidity through an inductive effect, giving rise to ionic interactions with the stationary phase. Indeed, the gas phase proton affinity for the chloro-analogue as calculated by combined DFT/ab initio approach was significantly smaller (331.3 kcal/mol) than for theophylline (340.9 kcal/mol), corroborating the existence of ionic bonds after abstraction of the proton in position 7 (Figure 8d and i). Hence, analytes eluted in predicted order when based on increasing surface polarity (blue area in Figure 8c–j).

In practical terms, the NP-like elution pattern precluded the use of the nonanalogous IS previously described for RP-LC-MS. Instead, the two closest eluting analogues, the ^{32}S-labeled 6-thiocaffeine and the stable isotope-labeled (SIL) $^{13}C_3$-caffeine were explored regarding their suitability as IS and compared to external standard calibration.

3.4 | Electrospray ionization–mass spectrometry

Since UV detection was insufficiently sensitive for the expected salivary levels after single oral application of 35 mg of caffeine, ESI coupled with single-quadrupole MS was explored as alternative to previously reported triple quadrupole MS/MS [50]. The makeup solvent choice and flow rate proved the major factors in S/N optimization, with 2-propanol outperforming methanol and lower flow rates benefiting sensitivity. Results further improved upon addition of water (2%) and formic acidic (0.75%) to aid ionization (detection in positive mode). A flow rate of 0.05 mL/min sufficed to prevent analyte precipitation within the ESI capillary upon CO_2 decompression and used as a compromise, although the highest S/N ratios were obtained without makeup. Among the remaining interface parameters, increasing the desolvation line temperature and the drying gas flow rate also significantly improved sensitivity (Figure 9).

3.5 | Validation

Off-line quantification of study samples by HPLC-MS/MS suggested caffeine levels between 50 and 1000 ng/mL during gastric emptying. Hence, an LLOQ of 31.25 ng/mL and an upper LOQ of 1000 ng/mL was set. Linearity, accuracy, and precision was compared for three calibration

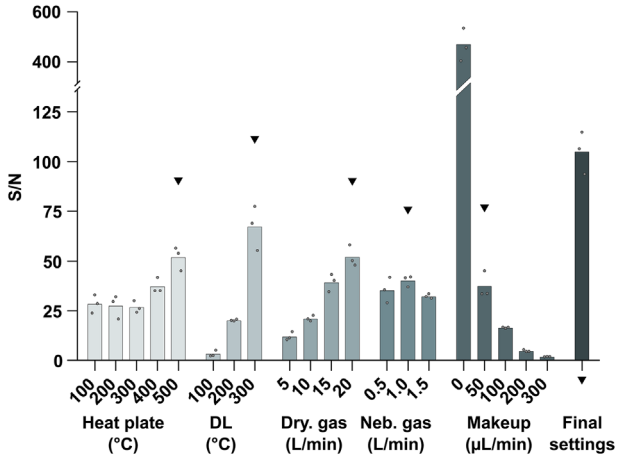

FIGURE 9 Optimization of MS parameters as measured by S/N. $n = 3$ (circles); injection volume: 5 µL; concentration: 100 ng/mL; SIM $[M+H]^+$; DL, desolvation line

models: (1) IS quantification using the SIL-IS $^{13}C_3$-caffeine, (2) IS quantification using the non-SIL-IS ^{32}S-analogue of the target analyte (6-thiocaffeine), and (3) without IS (i.e., external standard calibration) (Table 2).

Judging from calibrators, quantification based on the ^{32}S-labeled IS (2) appeared to yield reasonable results but revealed grave errors when confronted with QC samples (bias and RSD of up to 55.7% and 64.5%, respectively). When relying on external standard calibration only (3), accuracy and precision declined even further (bias and RSD of −96.4% and 67.2% at LLOQ). As shown in Table 2, the SIL-IS quantification model based on $^{13}C_3$-caffeine (1) provided accurate and precise results (<±20% at LLOQ; <±15% at all other levels) and was therefore pursued further.

As suggested by $R^2 \geq 0.9983$, a robust and reproducible linear relationship was corroborated by Mandel's fitting test [90]. Selectivity, that is, the ability to differentiate between the target analyte and interferences, was examined by comparing blank saliva from six healthy volunteers to LLOQ-spiked QCs. The final method passed the harmonized acceptance criteria set by FDA [53] and EMA [54] (signal ratio <20% for caffeine; <5% for IS). The same acceptance criteria were applied to confirm the absence of carry-over (analysis of a blank sample following a sample spiked to yield the upper LOQ (i.e., 1000 ng/mL) [54].

3.6 | Application

The SFE–SFC–MS method was applied to samples from a healthy volunteer of an MRI-validated gastric emptying study. The resulting salivary caffeine levels (SFE–SFC–MS) matched the gastric emptying data (MRI) in a healthy volunteer ($R^2 = 0.945$) in accordance with a previous study relying on off-line analysis (Figure 10) [48].

4 | DISCUSSION

Gastric emptying strongly affects the drug absorption profile of orally administered drugs, but data remain scarce since direct visualization requires advanced instrumentation such as MRI. Tracer drugs (whose plasma levels are limited almost exclusively by gastric emptying) allow gastric monitoring by standard chromatographic equipment but require invasive sampling when performed from plasma. A subset of these tracers can also be detected in saliva, which can be sampled noninvasively at little to no discomfort to the study participants but exposes staff to biomatrix and extraction solvents. Analysis of salivary tracers by SFE-SFC-MS combines noninvasive sampling with low-exposure analysis.

After passing the stomach, caffeine is readily absorbed, enters circulation, and is quickly secreted into the oral fluid. In comparison to previously reported determination methods from biological fluids, online SFE–SFC–MS required the least amount of sample preparation of all chromatographic methods, paralleled only by electrochemical methods such as square wave voltammetry that also exposed operators only during a single pipetting step to the salivary specimens (Table 3).

With manual handling accounting for three out of four LAIs (generally due to human error), the largely automatized SFE–SFC–MS method is an attractive alternative to conventional off-line techniques from a safety point of view [91]. In addition, reliance on CO_2 required no organic solvents during handling or extraction, facilitated lag-free transition to SFC, and supported flow rates of up to 3 mL/min due to the low viscosity of supercritical CO_2 (extraction and analysis within 10 min). While SFE-SFC-MS was utilized here to monitor gastric emptying in a single volunteer, these advantages can be applied to numerous physiological and pathophysiological situations, including TDM (e.g., caffeine in neonates), CYP1A2 phenotyping (caffeine metabolites), and determinations of other exogeneous or endogenous salivary tracers.

5 | CONCLUDING REMARKS

The aim of this study was to explore the utility of SFE–SFC–MS for the analysis of saliva. Taking the salivary marker caffeine as target analyte, the online approach yielded NP-like elution patterns that provided fast (10 min)

TABLE 2 Accuracy and precision of three different calibration models. HQC, high quality control; LQC, low quality control; MQC, medium quality control; values out of specification marked in bold

Model	Sample	Nominal (ng/mL)	Calculated (ng/mL)	Accuracy (%)	Precision (%)
SIL-IS $^{13}C_3$-caffeine ($n = 3$)	C1	31.3	27.3 ± 5.4	−12.7	17.4
	C2	62.5	59.0 ± 2.9	−5.6	4.7
	C3	125.0	129.3 ± 9.0	3.5	7.2
	C4	250.0	247.0 ± 3.7	−1.2	1.5
	C5	500.0	508.7 ± 26.6	1.7	5.3
	C6	1000.0	996.3 ± 13.0	−0.4	1.3
Within-run ($n = 6$)	LQC	62.5	59.0 ± 4.6	−5.6	7.4
	MQC	500.0	508.3 ± 35.2	1.7	7.0
	HQC	1000.0	955.2 ± 66.8	−4.5	6.7
Between-run ($n = 6$)	LQC	62.5	61.3 ± 6.2	−1.9	10.0
	MQC	500.0	486.2 ± 3.0	−2.8	0.6
	HQC	1000.0	918.8 ± 2.5	−8.1	0.3
					$R^2 = 0.9983$
Thio-IS ^{32}S-caffeine ($n = 3$)	C1	31.3	31.9 ± 0.6	1.9	1.9
	C2	62.5	48.9 ± 16.4	**−21.7**	**26.3**
	C3	125.0	115.8 ± 18.9	−7.3	**15.1**
	C4	250.0	257.9 ± 14.0	3.2	5.6
	C5	500.0	553.7 ± 63.7	10.7	12.7
	C6	1000.0	973.6 ± 29.9	−2.6	3.0
Within-run ($n = 3$)	LQC	62.5	65.8 ± 28.8	5.2	**46.0**
	MQC	500.0	671.0 ± 207.6	**34.2**	**41.5**
	HQC	1000.0	1454.9 ± 154.4	**45.5**	**15.4**
Between-run ($n = 3$)	LQC	62.5	77.3 ± 29.0	**23.6**	**46.5**
	MQC	500.0	738.3 ± 226.0	**47.7**	**45.2**
	HQC	1000.0	1557.3 ± 65.6	**55.7**	6.6
					$R^2 = 0.9819$
Without IS ($n = 3$)	C1	31.3	1.1 ± 21.0	**−96.4**	**67.2**
	C2	62.5	37.7 ± 19.6	**−39.6**	**31.3**
	C3	125.0	105.6 ± 12.4	**−15.5**	10.0
	C4	250.0	318.9 ± 10.4	**27.6**	4.2
	C5	500.0	535.3 ± 88.1	7.1	**17.6**
	C6	1000.0	970.0 ± 42.2	−3.0	4.2
Within-run ($n = 6$)	LQC	62.5	60.1 ± 14.6	−3.9	**23.4**
	MQC	500.0	563.1 ± 188.9	12.6	**37.8**
	HQC	1000.0	1038.8 ± 80.0	3.9	8.0
Between-run ($n = 6$)	LQC	62.5	66.8 ± 13.7	6.9	**21.9**
	MQC	500.0	541.4 ± 5.6	8.3	1.1
	HQC	1000.0	1056.9 ± 7.9	5.7	0.8
					$R^2 = 0.9732$

and robust results when adsorbed onto SiO_2, notably without the need for a drying step prior to extraction.

An SIL-IS was required to yield accurate and precise quantitative data (±20% at LLOQ; ±15% at higher concentrations). The makeup choice and flow rate proved the dominant factors in optimizing the S/N ratio, optimization of which allowed sensitive and selective detection by single-quadrupole MS in the range of 31.25–1000 ng/mL without the need for triple-quadrupole MS/MS. Applied to samples from an MRI-validated study, salivary

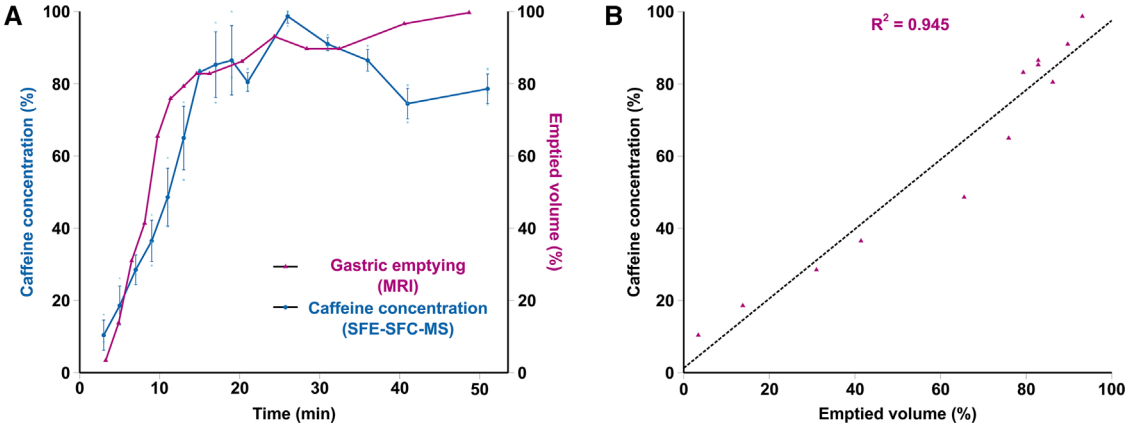

FIGURE 10 Caffeine concentrations match gastric emptying. (A) Normalized salivary caffeine concentrations ($c_{max} = 100\%$) versus normalized gastric emptying over time for a healthy volunteer ($n = 3$). (B) Correlation of normalized salivary caffeine concentrations (SFE-SFC-MS) versus normalized gastric emptying (MRI)

TABLE 3 Quantitative methods for caffeine from biological fluids

Exposure	Technique	Sample preparation	Biomatrix
High	ESI-IMS [92]	Protein precipitation, centrifugation, dilution	Plasma
	RPLC-UV [93]	SPE	Meconium
	CE-MS [94]	SPE	Urine
	Potentiometry [95]	Dilution and centrifugation; online single-drop liquid microextraction	Saliva
	RPLC-MS/MS [96]	Precipitation, centrifugation, SPE	Breast milk
	RPLC-MS/MS [97]	Decontamination, micronization, digestion, centrifugation, SPE	Hair
	RPLC-UV [98]	Centrifugation, dilution, SPE	Serum
Medium	ELISA [99]	Protein precipitation	Saliva
	Voltammetry [100]	Protein precipitation	Serum
	Micellar CE-UV [101]	Dilution and filtration	Serum
	RPLC-MS/MS [102]	Dilution and filtration	Urine
	RPLC-MS/MS [103]	Centrifugation, filtration, dilution	Saliva, plasma, urine
	Micellar RPLC-UV [104]	Filtration	Urine
	RPLC-UV [105]	Filtration	Urine
	Voltammetry [106]	Dilution/filtration	Urine, serum
	CE-UV [107]	Filtration	Urine, serum
	NMR [44]	Filtration	Saliva
Low	Voltammetry [108–110]	Sample introduction	Urine, serum
	SFE-SFC-MS	Sample introduction	Saliva

CE, capillary electrophoresis; ELISA, enzyme-linked immunosorbent assay; IMS, ion mobility spectrometry; NMR, nuclear magnetic resonance.

caffeine content as determined by SFE–SFC–MS correlated well with volumetric measurements of gastric emptying ($R^2 = 0.945$). Exploiting the possibility of participants sampling their own saliva, the operator was exposed only during a single pipetting step to biological specimens before online extraction and analysis took place. Overall, SFE–SFC–MS was among the least treatment-intensive bioanalytical methods reported for the given analyte.

The spreading of COVID-19 by aerosolized saliva has proven both the transmission risk as well as the potential diagnostic value of oral fluids. Although SFE-SFC-MS was applied here to noninfective samples only, the possibility to selectively extract low molecular weight analytes from polar matrix by an automated method suggests CO_2-based techniques to be a useful alternative when confronted with saliva of unclear or confirmed infection status. Protecting

operators' safety has been reliant on general safety precautions and personal protective equipment in the past. But safety-by-design may merit more focus than granted so far, demanding the same careful consideration as analytical correctness, speed, and environmental impact.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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