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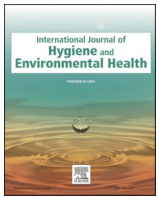

Validity of spot urine samples for estimating systemic fluoride exposure in children across diverse fluoridation modalities

Fatemeh Vida Zohoori [a,*], Marilia Afonso Rabelo Buzalaf [b], Anne Maguire [c], Roy Sanderson [d], Rodrigo A. Giacaman [e], Stefania Martignon [f], Edgar O. Beltran [f], Yaysa Vasquez [f], Fatemeh Eskandari [a], Jelena Kronic [a], Karla Gambetta-Tessini [e,g], Flavia Mauad Levy [b]

[a] School of Health and Life Sciences, Teesside University, Middlesbrough, UK
[b] Bauru School of Dentistry, University of Sao Paulo, Brazil
[c] School of Dental Sciences, Faculty of Medical Sciences, Newcastle University, UK
[d] School of Natural & Environmental Sciences, Newcastle University, UK
[e] Cariology Unit, Department of Oral Rehabilitation, Faculty of Dentistry, University of Talca, Talca, Chile
[f] UNICA – Caries Research Unit, Research Department, Universidad El Bosque, Bogotá, Colombia
[g] National Dental Research Institute Singapore, National Dental Centre Singapore, Singapore

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ABSTRACT

Objective: Reliable biomarkers are essential for assessing children's fluoride exposure, yet 24-h urinary fluoride excretion (24 h-UFE), the gold standard, is logistically challenging to collect. The validity of spot urine samples as a proxy across fluoridation modalities has not been evaluated. This study is the first to assess the predictive accuracy of spot urine fluoride concentration (U_{FC}), creatinine-adjusted ($U_{F/CR}$), and specific gravity-adjusted ($U_{F/SG}$) for estimating 24 h-UFE across multiple fluoride exposure modalities.

Methods: In this multi-modality observational study, 178 children aged 4–6 years residing in regions with different fluoride modalities were included: water fluoridation (UK, Brazil), salt fluoridation (Colombia), milk fluoridation (Chile), and non-fluoridated areas (UK, Chile). Each child provided one 24-h urine sample and four spot samples during a separate session (post-breakfast, post-lunch, before bedtime, first morning void). Linear regression models assessed the predictive validity of U_{FC}, $U_{F/CR}$, and $U_{F/SG}$.

Results: Mean urinary fluoride ranged 0.48–1.38 mg/L (U_{FC}), 1.13–2.30 mg/g ($U_{F/CR}$), and 0.55–1.70 mg/L ($U_{F/SG}$). UFC showed the strongest association with 24 h-UFE (mean $R^2 = 77\%$), particularly in water-fluoridated areas (up to 85%). $U_{F/CR}$ and $U_{F/SG}$ correlations were weaker (mean $R^2 = 58\%$ and 61%). Accuracy improved when multiple spot samples were used. Timing of peak fluoride excretion varied: post-breakfast (water), post-lunch (salt), first morning void (milk and non-fluoridated areas).

Conclusion: Spot U_{FC} provides a practical alternative for population-level monitoring of fluoride exposure in children, although accuracy depends on sampling time and fluoridation modality. This multi-modality study demonstrates variability in fluoride excretion across sources and informs optimized sampling strategies for public health surveillance.

1. Introduction

Dental caries remains a major global public health issue, with reports from 204 countries indicating a rise in the age-standardised incidence rate of untreated dental caries in both primary and permanent dentitions from 1990 to 2019 (Qin et al., 2022). Shifting focus from treatment to prevention is key to addressing this issue. Fluoride plays a vital role in preventing dental caries by enhancing enamel resistance and supporting the remineralisation process. Fluoridation schemes, used internationally, like water, salt, or milk aim to reduce dental caries while addressing dental health inequalities. As of 2015, nearly one billion people (17% of the global population) were reported to benefit from community-based

* Corresponding author.
E-mail address: v.zohoori@tees.ac.uk (F.V. Zohoori).

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fluoridation programs (U.S Public Health Service, 2015). However, a recent Cochrane review (Iheozor-Ejiofor et al., 2024) suggests that contemporary water fluoridation may provide benefits in children, though evidence remains limited for adults, disparities, and the effects of cessation, highlighting the importance of context-specific implementation and careful monitoring of fluoride exposure.

However, excessive fluoride intake, particularly during the early stages of tooth development, can increase the risk of dental fluorosis. To minimise this risk, total fluoride intake from all sources should remain below the Tolerable Upper Intake Level of 0.1 mg per kilogram of body weight per day (mg/kgbw/d), especially during the critical developmental period from one to eight years of age (EFSA Panel on Dietetic Products NAllergies, 2013; EFSA Scientific Committee et al., 2025; Institute of Medicine, 1997). The global consumption of ready-prepared foods and drinks has created a "halo" effect, where fluoridated products are transported to non-fluoridated areas and vice versa (Zohoori and Maguire, 2018). Given this cross-regional movement of fluoride-containing products, the World Health Organisation (WHO) (WHO, 2014) recommends careful monitoring of total fluoride exposure before and after the implementation of fluoridation schemes to ensure safety and effectiveness. However, the accurate measurement of individual fluoride intake from all sources, including diet and inadvertent toothpaste ingestion, is difficult and costly (Omid et al., 2016).

Urine is the primary excretion route for systemically absorbed fluoride, with most of an ingested dose appearing in the urine within the first 3 h and returning to baseline levels within 8 h (Whitford, 1994). While 24-h urinary fluoride excretion (24 h-UFE) provides an accurate estimate of daily fluoride intake in children (Villa et al., 2010), collecting a 24 h sample is logistically challenging, especially for non-toilet-trained children. Recent scoping and systematic reviews, including meta-analyses (Eskandari et al., 2023; Kumah et al., 2022), indicate that spot urine concentration may serve as a practical, non-invasive biomarker for population-level monitoring but it remains unclear whether it is suitable at an individual level. In addition, spot samples can be influenced by diurnal fluctuations in fluoride excretion, and adjustments for creatinine or urine specific gravity are often used to reduce this variability (Carrieri et al., 2001). Evidence from a UK study suggests that creatinine-adjusted spot urine concentration correlates well with daily fluoride intake, though findings are limited to a single location and water-fluoridation context (Zohoori and Maguire, 2017).

A pooled analysis of data from nine independent studies involving 212 children aged 0.15 to 7 years across six countries demonstrated that 24-h urinary fluoride excretion is a reliable biomarker of fluoride exposure (Villa et al., 2010). Building on this evidence, the present study used 24-h urinary fluoride excretion as the reference measure of fluoride intake and compared it with spot urine measurements, including urinary fluoride concentration (U_{FC}) as well as fluoride concentrations adjusted for specific gravity ($U_{F/SG}$) and creatinine ($U_{F/CR}$). This study evaluated these indices in children exposed to various fluoridation modalities, including community water, milk, and salt, across multiple geographic locations. Secondary objectives were to assess potential variations in 24 h-UFE and to determine the optimal timing for spot urine collection according to the specific fluoride exposure modality.

2. Material and methods

2.1. Study design and setting

This study employed a multi-centre observational design involving children exposed to different fluoride delivery modalities (salt, milk, and water) through established public health programs at various locations. The selection of study sites was purposive, with each location chosen to represent a distinct fluoridation scheme. This allowed for meaningful comparisons of urinary fluoride measures across different modalities and settings, reflecting real-world implementation of fluoride interventions in diverse contexts.

The research was conducted at six sites with different fluoridation modalities:

Middlesbrough, UK (Non-fluoridated, <0.3 mgF/L of drinking water) (NFW-UK)

San Clemente, Maule Region, Chile (Non-fluoridated, <0.3 mgF/L of drinking water) (NFW-Chile)

Hartlepool, UK (Water-fluoridated, 0.8-1.3 mgF/L of drinking water) (FW-UK)

Bauru, Brazil (Water-fluoridated, 0.8 mgF/L of drinking water) (FW-Brazil)

San Clemente and San Javier, Chile (Milk-fluoridated, 4.25 mg/L of powdered milk) (FM-Chile)

Bogotá, Colombia (Salt-fluoridated, 180-220 mg Potassium Fluoride (KF)/kg of salt; i.e. 59-72 mgF/kg of salt) (FS-Colombia).

2.2. Sample size determination

Sample size calculation was based on detecting significant correlations, using an upper reference value of 1.69 mg/g for $U_{F/CR}$ associated with high fluoride exposure (>0.07 mg/kg body weight/day) (Zohoori and Maguire, 2017). This calculation indicated that approximately 23 children per group would provide 80% power at a 0.05 significance level. To ensure sufficient power and allow for potential incomplete data, the study aimed to recruit 30 children per group. With six groups included in the study, this corresponded to an overall target sample size of 180 children, allowing examination of the relationships between 24 h-UFE and U_{FC}, $U_{F/SG}$ and $U_{F/CR}$ in spot urine samples. This target also aligned with WHO recommendations for fluoride exposure surveillance (WHO, 2014).

2.3. Ethical approvals

The study received ethical approvals from relevant Local Research Ethics Committees in the UK, Brazil, Chile, and Colombia. Written informed consent was obtained from the parents of the children recruited.

2.4. Study participants and procedures

The study included healthy children aged 4-6 years of both genders. Participants were required to be lifelong residents of the study area, free from metabolic or renal diseases, not using any other form of systemic fluoride, and not having received professionally applied topical fluoride treatments (e.g., varnishes or gels) in the past month. These eligibility criteria were assessed through a parent interview prior to the child's enrolment.

A standardised protocol for collecting data and biological samples (urine samples and participant questionnaires) was jointly designed by the collaborating research teams and applied uniformly across all study locations. Prior to data collection, all teams underwent training to ensure procedural consistency, and ongoing communication was maintained throughout the study period to address any challenges and support standardised implementation across sites.

Each child participated in two data and sample collection sessions, scheduled less than a week apart, on a date/day that was convenient for both parents and children, which could be on weekdays or weekends. In **Session A**, four spot urine (SU) samples were collected: 3 h after breakfast (SU1), 3 h after lunch (SU2), before bed on Day 1 (SU3), and first morning urine on Day 2 (SU4). In **Session B**, a 24-h urine sample was collected. Fig. 1 shows a schematic representation of the urine collection procedure. Participants were randomly assigned to their session order using a pre-prepared list. Sample collection involved three visits: **Visit 1** included measuring the child's weight and providing urine collection equipment with detailed instructions to ensure proper sample collection. Additionally, the child's toothbrushing habits were recorded, including the type of toothpaste used. The fluoride content of the

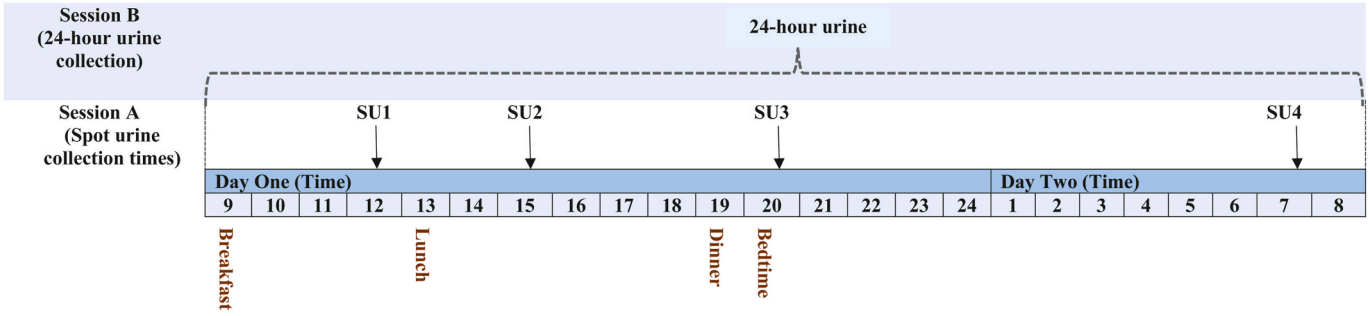

Fig. 1. A schematic representation of time intervals for spot and 24-h urine collections for children in Sessions A and B.

toothpaste was recorded from the product's labels. A sample of home tap water was also collected. **Visit 2** involved collecting either 24-h or spot urine samples; and **Visit 3**, conducted almost 7 days after Visit 2, involved collecting the second set of samples. Mealtimes (breakfast, lunch, and dinner), along with bedtime and morning wake time, were recorded by the parents.

Additionally, dietary intake was assessed using a parent-completed 24-h dietary recall, supported by detailed instructions and a follow-up interview to ensure complete and accurate recording of all foods and beverages, including water and milk, consumed by the child (Zohoori et al., 2025).

2.5. Urine and water sample preparation and analysis

Fluoride concentrations in urine and water samples were measured in triplicate at room temperature using an fluoride-ion-selective electrode (F-ISE Model 79609; Orion Research) and a potentiometer (Model 720 A), following a direct analysis method with TISAB III (Martínez-Mier et al., 2011). Creatinine concentrations were assessed using the Jaffe method (Bones and Taussky, 1951) and an ADVIA 1650 autoanalyzer (Siemens Medical Solutions Diagnostics). Urine specific gravity, determined as the ratio of urine density to distilled water density, was measured with a handheld digital refractometer (Minton et al., 2015).

To validate the analytical methods and ensure consistency across sites, Fluoride Urine Standard Reference Materials (FU-SRM1805 and FU-SRM1815) were also obtained from the Institut National de Santé Publique du Québec, Canada, and analysed by all participating laboratories. In addition, the robustness of the analytical approach was verified through the re-analysis of 10% of the samples. Both the initial and repeat measurements were conducted in triplicate to ensure reliability and precision.

The validity of the U_{SG} test was verified by measuring the specific gravity of water (1.0 at 4 °C), which served as a control. The creatinine measurements were conducted in certified laboratories within hospitals at each location where external quality control measures were in place.

2.6. Outcome measures and calculations

2.6.1. Calculation of 24-h urinary fluoride excretion (24-h UFE)

Daily urinary fluoride excretion (DUFE; mg/day) was calculated by multiplying the total urine volume (L) collected over the day by the fluoride concentration of the sample (mg/day). The collection period began at the child's wake-up on day one and ended at wake-up on day two. To account for variations in collection duration, DUFE was normalised to a 24-h period (24-UFE; mg/24 h) using the formula:

$$\text{24-UFE (mg/24h)} = \text{DUFE (mg/day)} \times [24/\text{collection duration (hours)}]$$

Finally, 24-UFE was divided by the child's body weight to express fluoride excretion per kilogram (24-UFE; mg/kg bw/24 h), allowing comparability across participants.

2.6.2. Calculations of $U_{F/CR}$ and $U_{F/SG}$ in spot urine sample

$U_{F/CR}$ was calculated by dividing the fluoride concentration (mg/L) by the creatinine concentration (g/L) in urine, expressed in mg/g creatinine (Zohoori and Maguire, 2017):

$$\mathbf{U_{F/CR}}(\text{mg/g}) = \mathbf{U_{FC}}(\text{mg/L})/\mathbf{U_{CR}}(\text{g/L})$$

$\mathbf{U_{F/SG}}$ for each urine sample was calculated using the following formula (Hauser et al., 2004; Thomas et al., 2016; Till et al., 2018):

$$\mathbf{U_{F/SG}}(\text{mg/L}) = \mathbf{U_{FC}}(\text{mg/L}) \text{x} [(SG_m - 1)/(SG_i - 1)],$$

where $\mathbf{U_{FC}}$ is the measured fluoride concentration in urine, SG_i is the measured specific gravity of each urine sample and SG_m is the median specific gravity for the study group.

2.7. Statistical analysis

To evaluate the potential of spot urine measurements as predictors of 24 h-UFE, a series of linear regression models were constructed for U_{FC}, $U_{F/SG}$ and $U_{F/CR}$, with R^2 values reported to assess model fit. Models were run separately for each study site and fluoridation modality to ensure that predictive performance was assessed within comparable exposure contexts. Separate models were run for each SU sample (1, 2, 3, or 4 measurements) to examine whether the timing and number of spot urine collections influenced the predictive ability of these metrics. Model assumptions were assessed through quantile-quantile (QQ) normality plots of residuals, and no violations were observed.

One-way ANOVA was used to assess differences in 24 h-UFE, U_{FC}, $U_{F/SG}$ and $U_{F/CR}$ across fluoridation modalities and spot urine collection times, with Tukey's *post hoc* test applied for pairwise comparisons where ANOVA results were significant to control for multiple testing.

Pearson correlation coefficients were calculated to assess associations between unadjusted urinary fluoride (UF), UF adjusted for specific gravity (UF/SG) and creatinine (UF/CR), and water fluoride concentrations. Due to minimal variability in water fluoride concentrations within fluoridation modalities, all samples were pooled across modalities for this analysis, allowing correlations to primarily reflect variation between fluoridation modalities rather than individual-level differences in exposure.

3. Results

3.1. Validity of the analytical method

The measurement of the fluoride concentration of FU-SRMs from all laboratories fell within the accepted range of 0.366-0.390 mg/L for FU-SRM1805 and 0.601-0.628 mg/L for FU-SRM1815.

The mean (SD) fluoride concentration measured in FU-SRM1805 was 0.384 (0.003) mg/L, and for SRM-1815, it was 0.613 (0.008) mg/L. These results were within the certified reference ranges for each standard (0.366–0.390 mg/L for FU-SRM1805 and 0.601–0.628 mg/L for SRM-1815), supporting the reliability and consistency of the analytical

procedures used across laboratories.

To further assess analytical accuracy, 10% of the samples were re-analysed. The repeated measurements showed no statistically significant differences from the originals ($p = 0.426$), with a mean difference of 0.003 ± 0.02 mgF/L, demonstrating strong reproducibility of the method.

3.2. Characteristics of the study participants

Table 1 shows participant metrics, e.g. toothbrushing frequency, fluoride concentration of toothpaste, mealtimes, and bed- and wake-time of the children who participated in the study. The study involved 178 children, with ages ranging from 5.0 years in NFW-UK and FS-Colombia to 6.2 years in FM-Chile, and weights ranging from 17.0 kg in FS-Colombia to 28.5 kg in FM-Chile. The overall proportion of males was slightly higher (51.7%), ranging from 43.3% in NFW-Chile to 73.3% in FM-Chile. Toothbrushing frequency ranged from 2.2 to 3.2 times/day, and fluoride concentration in toothpaste ranged from 1188 to 1500 μg/g. Mealtimes varied from 8:20-9:50 for breakfast, 12:30-13:40 for lunch, and 16:30-19:45 for dinner.

3.3. 24-h urinary fluoride excretion (24-h UFE)

Mean (SD) values for daily urine volume, urinary fluoride excretion (both unadjusted and adjusted for body weight), and creatinine levels in children, categorised by fluoridation modality, are provided in Supplementary Material (SM) – Table 1.

The median, interquartile range, and outlier values for 24 h-UFE by modality are presented in Fig. 2.

Table 2 shows the summary of mean 24-h UFE, adjusted for body weight, by fluoridation modality. The mean (SD) 24-h UFE (adjusted for body weight) of the children was: 0.018 (0.015) mg/kgbw/d in NFW-UK, 0.035 (0.017) mg/kgbw/d in FW-UK, 0.023 (0.009) mg/kgbw/d in FW-Brazil, 0.025 (0.013) mg/kgbw/d in FS-Colombia, and 0.019 (0.010) mg/kgbw/d in both groups in Chile (NFW-Chile, and FM-Chile). Within each fluoridation modality, the FW-UK group exhibited the highest variation in 24 h-UFE at the individual level. Additionally, the mean 24 h-UFE in the FW-UK group was significantly higher ($p < 0.05$) than in the non-fluoridated water groups (NFW-UK and NFW-Chile), as well as the FW-Brazil and MF-Chile groups.

3.4. Spot urine samples

In total, 699 spot urine samples were collected from 176 children. Details on the number of collected spot urine samples, along with urinary fluoride and creatinine concentrations, as well as the adjusted urinary fluoride concentrations for creatinine and specific gravity by fluoridation modality, are provided in SM-Table 2. A summary of the statistically significant pairwise differences in U_{FC}, $U_{F/CR}$, and $U_{F/SG}$ derived from the full dataset in SM-Table 2 is presented in Table 2. Overall, the mean (SD) U_{FC} ranged from 0.480 (0.170) mg/L for the NFW-Chile group to 1.380 (0.680) mg/L for the FW-UK group. The mean (SD) $U_{F/CR}$ ranged from 1.130 (1.380) mg/g for the NFW-Chile group to 2.300 (2.510) mg/g for the FS-Colombia group. The mean (SD) $U_{F/SG}$ ranged from 0.550 (0.320) mg/L for the NFW-Chile group to 1.700 (0.970) mg/L for the FW-UK. The standard deviations observed within each fluoride modality group highlight significant variability in urine fluoride metrics (U_{FC}, $U_{F/CR}$ and $U_{F/SG}$) at the individual level. For instance, U_{FC} values, at the individual level, exhibited wide ranges across groups: 0.118–4.696 mg/L in NFW-UK, 0.230–1.000 mg/L in NFW-Chile, 0.373–3.447 mg/L in FW-UK, 0.340–2.500 mg/L in FW-Brazil, 0.280–1.450 mg/L in FM-Chile, and 0.125–4.418 mg/L in SF-Colombia.

Fig. 3 illustrates the trend in U_{FC}, $U_{F/SG}$ and $U_{F/CR}$ across four time points. The peak U_{FC} values (Fig. 3a) for the non-fluoridated groups (NFW-UK, NFW-Chile) and FM-Chile occurred at SU4 (fasting; i.e., the first voided urine in the morning on Day 2). For FS-Colombia, the peak was at SU2 (3 h after lunch on Day 1). In both water-fluoridation schemes (FW-Brazil and FW-UK), the peak U_{FC} was at SU1 (3 h after breakfast on Day 1). In both water-fluoridation schemes (FW-Brazil and FW-UK), the $U_{F/SG}$ (Fig. 3b) was relatively constant throughout the day. However, the peak $U_{F/SG}$ values for both groups in Chile (NFW-Chile and FM-Chile) were observed at SU3 (last urine before going to bed). For both FS-Colombia and NFW-UK, the peak was at SU2. In both water-fluoridation schemes (FW-Brazil and FW-UK), as well as in the non-fluoridated water scheme in the UK (NFW-UK), the peak $U_{F/CR}$ (Fig. 3c) was observed at SU1. For salt fluoridation (FS-Colombia) and non-fluoridated water in Chile (NFW-Chile), the peaks occurred at SU2. For the milk-fluoridation scheme (FM-Chile), the peak was observed at SU3.

The ANOVA model revealed no statistically significant interaction between fluoridation modalities and time of collection. Consequently, the main effects of modalities and time of collection of urine samples were examined separately.

Results of the post-hoc analysis including mean differences and 95% confidence intervals (CI) for overall U_{FC}, $U_{F/CR}$, and $U_{F/SG}$ between different fluoridation modalities are presented in detail in SM-Table 3. In summary, the pattern of differences between fluoridation modalities

Table 1
Mean (SD) anthropometric characteristics, toothbrushing frequency, fluoride concentration of toothpaste, meal times, and bed-time and wake-time of the children who participated in the study.

Variable	Fluoridation modality[a]					
	NFW-UK (n = 32)	NFW-Chile (n = 30)	FW-UK (n = 29)	FW-Brazil (n = 21)	FM-Chile (n = 30)	FS-Colombia (n = 36)
Fluoride concentration of supply water (mg/L)	0.11 (0.03)	0.07 (0.0)[a]	1.22 (0.06)	0.80 (0.01)	0.07 (0.0)[a]	0.03 (0.01)
Age (years)	5.0 (0.8)	5.6 (0.7)	5.7 (0.8)	5.1 (0.9)	6.2 (3.0)	5.0 (0.5)
Weight (kg)	20.1 (4.7)	24.2 (5.8)	21.1 (4.5)	20.0 (4.6)	28.5 (6.7)	17.0 (1.8)
Female: Male (number)	16:16	17:13	14:15	12:9	8:22	19:17
Frequency of toothbrushing per day	3.0 (0.3)	2.4 (0.6)	3.2 (0.5)	2.2 (0.4)	2.5 (0.6)	2.8 (0.5)
Fluoride concentration of toothpaste (μg/g)	1500 (0)[b]	1188 (178)[c]	1400 (193)[c]	1354 (159)[c]	1327 (179)[c]	1500 (0)[b]
Mealtimes						
Breakfast	08:20	09:30	08:30	09:00	09:50	08:30
Lunch	12:30	13:30	12:30	12:00	13:45	13:40
Dinner	17:30	19:30	16:30	21:00	19:45	19:30
Bedtime	19:45	21:50	20:10	22:30	21:15	21:15
Morning wake time	07:25	08:30	07:15	08:45	07:30	07:15

[a]Non-fluoridated water (NFW), Fluoridated water (FW), Fluoridated milk (FM), Fluoridated salt (FS).
[a](SD) rounded to 0.0; the value was <0.005 mg/L.
[b]All children used toothpaste containing the same fluoride concentration, as indicated on the product labels.
[c]Mean values. Children used toothpastes containing 1100–1500 ppm fluoride, as indicated on product labels.

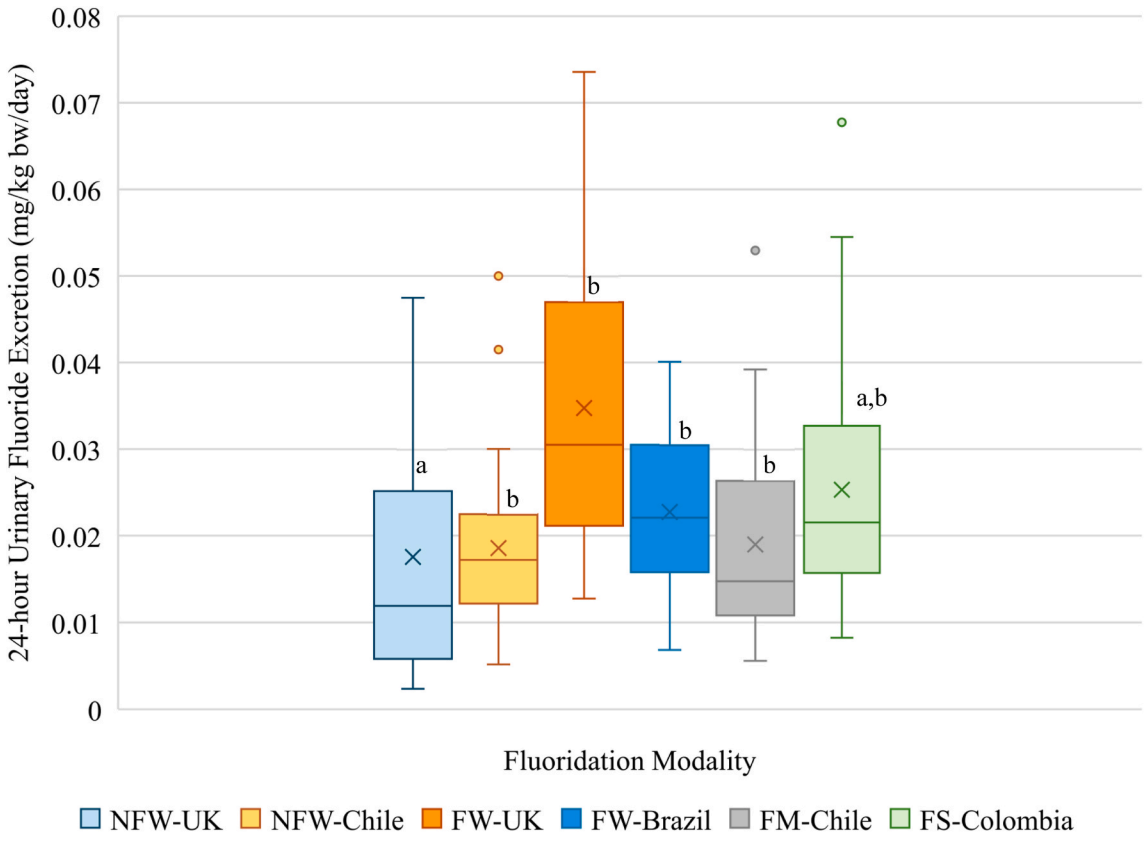

Fig. 2. Boxplot of 24-h urinary fluoride excretion (24 h-UFE, mg/kg bw/day) by fluoridation modality/area. The horizontal lines drawn inside the boxes denote the median of the 24 h-UFE; 'x' mark inside each box indicates the mean 24 h-UFE, lower and upper whiskers are 25th and 75th percentiles; bars represent 10th and 90th percentiles and dots denote outlier values. Different letters indicate statistically significant differences ($p < 0.05$) in 24 h-UFE between the schemes based on a Tukey test.
FW: Fluoridated water, NFW: non-fluoridated water, FM: Fluoridated milk, FS: fluoridated salt.

Table 2
Mean (SD) 24-h urinary fluoride excretion and urinary parameters in spot urine samples by fluoridation modality.

Variable	Fluoridation modality*					
	NFW-UK	NFW-Chile	FW-UK	FW-Brazil	FM-Chile	FS-Colombia
24-h urine						
Number of 24-h urine samples	32	30	29	21	30	36
Urinary fluoride excretion (UFE; mg/kg/24hr)	0.018 (0.015)	0.019 (0.010)	0.035 (0.017)	0.023 (0.009)	0.019 (0.011)	0.027 (0.014)
Spot urine: urinary parameters						
Total number of spot urine samples	117	120	115	83	120	144
Fluoride concentration (U_{FC}; mg/L)	1.160 (0.941)	0.482 (0.173)	1.380 (0.684)	0.947 (0.438)	0.586 (0.193)	1.252 (0.681)
Adjusted U_{FC} for U_{CR} ($U_{F/CR}$; mg/g)[i]	1.857 (1.585)	1.131 (1.382)	2.261 (1.597)	2.268 (2.593)	1.141 (1.044)	2.295 (2.510)
Adjusted U_{FC} for U_{SG} ($U_{F/SG}$; mg/L)[ii]	1.466 (1.604)	0.547 (0.323)	1.703 (0.969)	0.929 (0.567)	0.791 (0.560)	1.473 (1.202)

*Non-fluoridated water (NFW), Fluoridated water (FW), Fluoridated milk (FM), Fluoridated salt (FS).
[i]**Adjusted $U_{F/CR}$** (mg/g) = **U_{FC}** (mg/L)/**U_{CR}** (g/L); where **U_{FC}** is the measured fluoride concentration in urine and **U_{CR}** is the measured creatinine concentration in urine.
[ii]**Adjusted $U_{F/SG}$** (mg/L) = **U_{FC}** (mg/L) x $[(SG_m - 1)/(SG_i - 1)]$; where **U_{FC}** is the measured fluoride concentration in urine, SG_i is the measured specific gravity of each urine sample and SG_m is the median specific gravity for the cohort.

was largely consistent across all three measurements, although the statistical significance of individual comparisons varied by outcome. Several modality pairs, including FM-Chile vs NFW-Chile, FW-UK vs NFW-UK, and FS-Colombia vs NFW-UK, showed no statistically significant differences across any of the measures, while others were non-significant for one or two outcomes. In contrast, multiple comparisons demonstrated statistically significant differences for at least one of the three fluoride metrics.

Regarding the time of collection, post-hoc analysis indicated that U_{FC} at SU4 was significantly ($p < 0.001$) higher than at other time points for both Chilean groups (NFW-Chile and FM-Chile) (Fig. 3a). Additionally, in the NFW-Chile group, $U_{F/SG}$ at SU3 was significantly higher than at SU1 ($p < 0.001$) and SU2 ($p = 0.005$) (Fig. 3b). However, the time of urine collection did not produce statistically significantly difference in U_{FC}, $U_{F/SG}$ and $U_{F/CR}$ for other modalities. These results suggest that while fluoridation modality is associated with the magnitude of urinary fluoride, the influence of collection time is modality-specific.

Table 4 presents the Pearson correlations between urinary fluoride measures. Water fluoride concentrations exhibited minimal variability within modalities, so all samples were pooled across fluoridation modalities. Urinary fluoride adjusted for specific gravity and for creatinine were strongly correlated with unadjusted urinary fluoride ($r = 0.82$ and 0.65, respectively; $p < 0.001$), while water fluoride concentration showed no significant correlation with any spot urinary fluoride

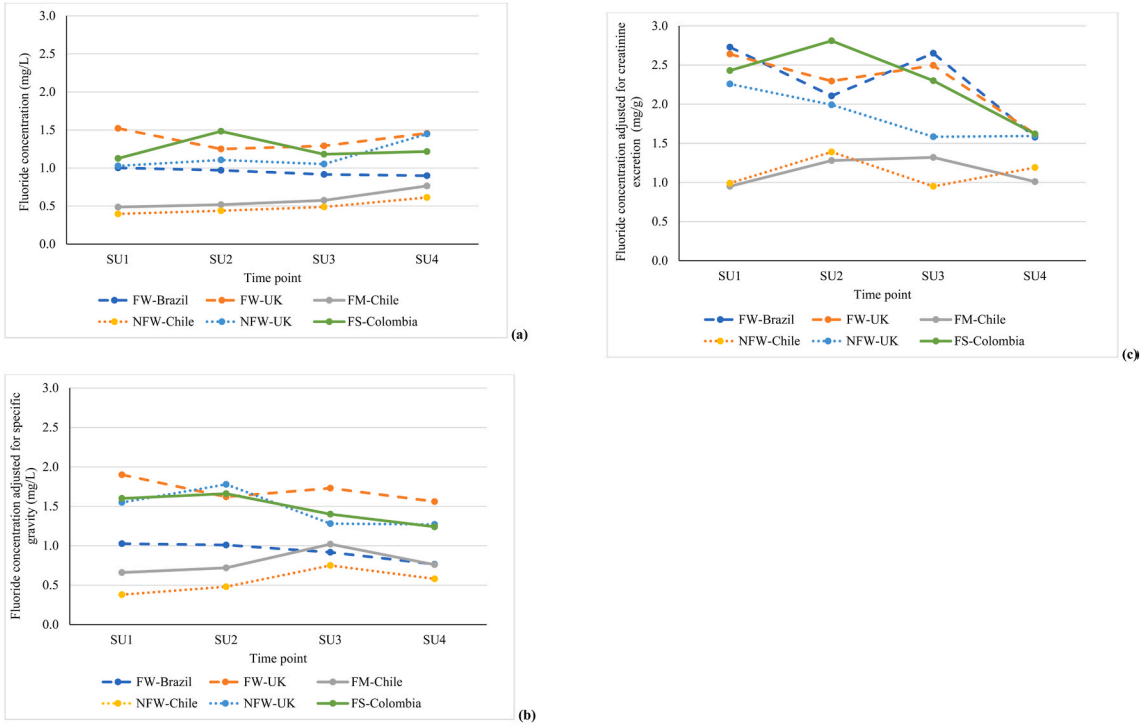

Fig. 3. (a) Fluoride concentration (U_{FC}, mg/L), of spot urine (SU) samples by fluoride modality **(b)** U_{FC} adjusted for specific gravity ($U_{F/SG}$, mg/L), and **(c)** U_{FC} adjusted for creatinine excretion ($U_{F/CR}$; mg/g).
(SU1) 3 h after breakfast on Day 1, (SU2) 3 h after lunch on Day 1, (SU3) last urine before going to bed on Day 1, and (SU4) fasting (first voided urine in the morning) on Day 2.
FW: Fluoridated water, NFW: non-fluoridated water, FM: Fluoridated milk, FS: fluoridated salt.

parameters (all $p > 0.05$).

3.5. Prediction of 24 h-UFE from spot urine (SU)

All linear models between 24 h-UFE and U_{FC}, $U_{F/CR}$ and $U_{F/SG}$ across combinations of one, two, three, or four spot urine samples for all fluoride modalities (including R^2 and p-values) are detailed in SM-Tables 4a to 4c and summarised in Tables 3a–3c. While correlations between 24 h-UFE and spot urine metrics (U_{FC}, $U_{F/CR}$ and $U_{F/SG}$) for these combinations were highly significant ($p < 0.001$) (SM-Tables 4a to 4c), variability across modalities was observed, as indicated by the mean R^2 estimates (Tables 3a–3c). Notably, the association between U_{FC} in spot urine and 24 h-UFE improved with an increasing number of spot samples, while for $U_{F/CR}$ and $U_{F/SG}$, increasing the number of samples did not enhance the prediction.

The accuracy, as measured by the R^2, explained approximately 66% to 88% of the variation in U_{FC} across all fluoridation modalities. In contrast, it explained 35% to 45% of the variation in $U_{F/CR}$ in salt fluoridation and 50% to 76% in the other modalities. Additionally, it accounted for 23% to 48% of the variation in $U_{F/SG}$ for fluoridated salt use and 50% to 82% in the other fluoride modalities.

4. Discussion

This multi-centre observational study represents the first comprehensive evaluation of three spot urinary fluoride indicators (urinary fluoride concentration, and urinary fluoride adjusted for specific gravity or creatinine) against 24-h urinary fluoride excretion in children exposed to diverse fluoride modalities across varied geographic settings. By directly comparing these indicators, the study provides clear evidence on their relative performance under real-world exposure conditions. The findings reaffirmed that 24 h-urinary fluoride excretion remains the most reliable reference method for assessing fluoride exposure at the population level. At the same time, the results demonstrate that spot urine samples can offer a practical alternative for population-level surveillance purposes. However, their validity is highly dependent on appropriate timing of sample collection and careful consideration of the specific exposure modality. While no standardised guidance currently exists for optimal spot urine sampling, our results indicate that timing should consider fluoride intake sources and patterns., and as such could help inform the development of such guidance.

Our findings show that, regardless of the modality of fluoridation, U_{FC}, $U_{F/CR}$ and $U_{F/SG}$ derived from spot urine samples could predict 24-h urinary fluoride excretion with average accuracies of 77%, 58%, and 61%, respectively. When examined within individual fluoridation modalities, accuracy was highest for U_{FC} in communities with fluoridated water (80% in the UK; 85% in Brazil), and lowest for $U_{F/CR}$ and $U_{F/SG}$ in the fluoridated-salt modality (40% and 38%, respectively; Table 3).

The interpretation and public health relevance of these findings can be considered in relation to the WHO's established guidelines for urinary fluoride excretion (WHO, 2014). According to these benchmarks, a 24-h UFE of 0.497 and $\geq$ 0.697 mg/d correspond to upper optimal (0.07 mg/kg BW/d) and high fluoride exposure ($\geq$0.1 mg/kg BW/d), respectively, for children aged four to seven years. The relatively small difference between these thresholds indicates that a prediction accuracy exceeding 60% for 24-h UFE would be sufficient to distinguish between "optimal" and "high" fluoride intake at the population level.

Against this threshold, our results suggest that creatinine- and specific gravity–adjusted measures in spot urines may not achieve adequate accuracy to consistently predict 24-h urinary fluoride excretion across different fluoridation modalities. In contrast, among the three spot urine parameters evaluated (U_{FC}, $U_{F/CR}$ and $U_{F/SG}$), unadjusted spot urinary fluoride concentration showed relatively strong predictive performance (particularly in water-fluoridated settings) and may serve as a practical option for population-level surveillance, although its validity remains highly dependent on appropriate timing of collection and the specific

Table 3
Mean R^2 between **(a)** fluoride concentration ($U_{FC,}$ mg/L), **(b)** U_{FC} adjusted for specific gravity ($U_{F/SG,}$ mg/L), and **(c)** U_{FC} adjusted for creatinine excretion ($U_{F/CR,}$ mg/g) of combinations of one, two, three, or four spot urine (SU) samples and 24-h urinary fluoride excretion (24 h-UFE) by modality.

(3a). U_{FC} (mg/L)

Number of SU samples	Fluoridation modality[a]					
	NFW-UK	NFW-Chile	FW-UK	FW-Brazil	FM-Chile	FS-Colombia
1	0.659	0.726	0.751	0.784	0.733	0.719
2	0.707	0.730	0.798	0.845	0.744	0.779
3	0.727	0.731	0.817	0.870	0.749	0.800
4	0.736	0.732	0.826	0.883	0.751	0.811
Average	0.707	0.730	0.798	0.846	0.744	0.777

(3 b). $U_{F/CR}$ (mg/g)

Number of SU samples	Fluoridation modality[a]					
	NFW-UK	NFW-Chile	FW-UK	FW-Brazil	FM-Chile	FS-Colombia
1	0.721	0.531	0.765	0.545	0.574	0.416
2	0.719	0.519	0.726	0.607	0.529	0.354
3	0.723	0.540	0.676	0.640	0.510	0.384
4	0.695	0.577	0.612	0.662	0.500	0.447
Average	0.715	0.542	0.695	0.614	0.528	0.400

(3c). $U_{F/SG}$ (mg/L)

Number of SU samples	Fluoridation modality[a]					
	NFW-UK	NFW-Chile	FW-UK	FW-Brazil	FM-Chile	FS-Colombia
1	0.600	0.630	0.795	0.720	0.610	0.481
2	0.544	0.632	0.739	0.782	0.581	0.478
3	0.554	0.659	0.700	0.809	0.549	0.349
4	0.635	0.668	0.674	0.824	0.505	0.227
Average	0.583	0.647	0.727	0.784	0.561	0.384

[a] FW: Fluoridated water, NFW: non-fluoridated water, FM: Fluoridated milk, FS: fluoridated salt.

modality of exposure. Importantly, adjusting urinary fluoride for specific gravity or creatinine was strongly correlated with unadjusted values (Table 4), suggesting that these adjustments do not substantially alter the ranking or relative exposure across participants. In contrast, water fluoride concentration showed no significant linear association with urinary fluoride measures, indicating that water fluoride may not be the sole source of fluoride exposure and that other factors (e.g. unintentional toothpaste ingestion) likely contribute to urinary fluoride levels.

Several factors may underlie the poorer performance of creatinine- and specific gravity–adjusted spot urine fluoride concentrations compared to unadjusted values when predicting 24-h urinary fluoride excretion. Although adjustment for creatinine or urine specific gravity has been proposed to mitigate the effects of urinary dilution and diurnal fluctuations (Carrieri et al., 2001), these correction methods are subject to important limitations. Creatinine and specific gravity are indirect proxies for urine dilution rather than direct markers of the physiological processes governing fluoride kinetics (such as intake timing, urinary flow rate, and renal clearance) so their use may introduce rather than remove variance. In our study, urinary creatinine concentrations, and consequently creatinine-adjusted urinary fluoride concentrations, showed greater variability across the four collection time points than specific gravity–adjusted urinary fluoride excretion, consistent with previous reports (Middleton et al., 2016; Wettersten et al., 2021). This increased variability reflects the fact that creatinine excretion fluctuates considerably over the course of the day and is influenced by age, sex, muscle mass, diet (particularly protein intake), renal function and ethnicity, contributing additional variability and potential bias (Gounden et al., 2024), whereas specific gravity is less affected by these factors. Consequently, correcting for creatinine may introduce more fluctuation in urinary fluoride measurements than specific gravity, particularly when spot samples are collected at varying times. Our findings, consistent with the literature (Pearson et al., 2009; Sallsten and Barregard, 2021), suggest that sample timing is critical; for example, first morning voids (overnight samples) typically have higher creatinine concentrations, and standardising the collection time may help reduce variability when creatinine adjustment is applied.

Table 4
Pearson correlation coefficients between spot urinary fluoride parameters[a] and water fluoride concentration WFC (mg/L).

	$U_{F/SG,}$ mg/L	$U_{F/CR,}$ mg/g	WFC
$U_{FC,}$ mg/L	0.82 ($P < 0.001$)	0.65 ($P < 0.001$)	0.10 ($P = 0.199$)
$U_{F/SG,}$ mg/L		0.74 ($P < 0.001$)	0.14 ($P = 0.056$)
$U_{F/CR,}$ mg/g			0.02 ($P = 0.808$)

[a] Fluoride concentration ($U_{FC,}$ mg/L), U_{FC} adjusted for specific gravity ($U_{F/SG,}$ mg/L), U_{FC} adjusted for creatinine excretion ($U_{F/CR,}$ mg/g).

Specific gravity, while also influenced by solutes other than fluoride (e.g., glucose, proteins, electrolytes) and by environmental and physiological factors such as hydration status, dietary composition, and altitude (Palubiski et al., 2020), appears to provide a more stable adjustment in repeated or varied-timing collections. In our study, this was reflected by the consistently higher urine specific gravity observed among children residing in Bogotá, Colombia (mean altitude 2640 m), compared with children from the other study sites. When exposure patterns differ across fluoridation modalities and spot samples fail to capture the timing of peak fluoride excretion, such simplistic dilution adjustments may not fully reflect true physiological variation. These considerations highlight the trade-offs between creatinine and specific gravity adjustment and reinforce the importance of careful interpretation of urinary fluoride biomarkers in children. Furthermore, empirical evidence further suggests that, for some analytes, unadjusted concentrations may correlate better with reference measures (Middleton et al., 2016; Wettersten et al., 2021).

An *a priori* assumption might be that increasing the number of spot urine samples would proportionally enhance the accuracy of predicting 24-h urinary fluoride excretion. However, the present findings indicate that such improvements were observed only for urinary fluoride concentration. Across all fluoridation modalities, the predictive accuracy of urinary fluoride concentration increased from a mean of 73% with a single spot urine sample to 79% when four samples were averaged. In contrast, no consistent improvement was observed for creatinine- or specific gravity–adjusted measures, likely reflecting the limitations of these adjustment methods discussed above.

In both the UK and Brazil, where water fluoridation was the primary modality, all three urinary parameters measured in spot urine (i.e. U_{FC} $U_{F/CR}$ and $U_{F/SG}$) peaked approximately 3 h after breakfast. By contrast, in Colombia, where children were exposed to fluoridated salt, the peak occurred 3 h after lunch, coinciding with the timing of their main meal prepared with fluoridated salt. These findings are consistent with the expected excretion kinetics, as the majority of ingested fluoride is eliminated in urine within the first 3 h following ingestion (Whitford, 1994). However, for the other fluoridation modalities, the timing of peak urinary fluoride concentration was not consistent across urinary parameters. Among children in the UK and Chile without any community fluoridation scheme, the highest values based on unadjusted urinary fluoride concentrations (U_{FC}) were observed in the first morning urine following overnight fasting (SU1). In contrast, when concentrations were adjusted for creatinine or specific gravity, the peaks occurred at later sampling times. For example, in the NFW-UK group, the highest specific gravity–adjusted values ($U_{F/SG}$) were observed at SU2, whereas the creatinine-adjusted ($_{F/CR}$) peak occurred at SU1. This discrepancy highlights the influence of adjustment methods on the interpretation of temporal patterns in urinary fluoride excretion.

Interestingly, despite the substantial difference in water fluoride levels between the fluoridated and non-fluoridated UK modalities (0.11

vs. 1.22 mg/L), spot urinary fluoride concentrations did not differ significantly between these groups. This likely reflects the combined influence of sample timing, recent fluoride intake, and daily variability, which can obscure differences at the individual or modality-specific level. While spot samples can capture periods of peak excretion, they may not reliably represent overall fluoride exposure or differentiate between exposure modalities.

When employing spot urine sampling, it has been recommended (Waterhouse et al., 1980) to include overnight fasting samples, as they provide a useful indicator of chronic fluoride exposure or body fluoride burden. Moreover, overnight samples more closely approximate 24-h collections, as urine is retained in the bladder for an extended period during sleep.

Collectively, these observations highlight both the utility and the limitations of spot urine sampling. While spot U_{FC}, particularly when collection is appropriately timed, based on the particular modality or fluoride source, emerges as the most practical and informative indicator for population-level monitoring, the substantial within-group variability observed across all fluoridation modalities indicates that spot urine samples are not suitable for reliable assessment of fluoride exposure at the individual level.

Overall, 24-h UFE in children was significantly lower in the Brazilian water-fluoridated area compared with the UK water-fluoridated area, reflecting differences in the fluoride concentration of the drinking water (0.80 mg/L in Brazil vs. 1.22 mg/L in the UK) and possibly variations in dietary patterns and oral hygiene practices. In contrast, in Chile, 24-h UFE did not differ significantly between children in the milk-fluoridation group and those in the non-fluoridated water group. This lack of difference may be explained by the high calcium content of milk, which can reduce fluoride absorption by up to 13% (Shulman and Vallejo, 1990), or by the influence of inadvertent fluoride intake from toothpaste, which can substantially contribute to total fluoride exposure and generally contains a higher fluoride concentration than milk. Overall, although 24-h UFE accounts for total urine volume and some daily variation in fluoride intake, a single collection cannot reliably reflect individual-level exposure, as excretion is influenced by day-to-day variability in diet, hydration, environmental temperature, and physical activity, all of which can affect fluid consumption and urinary fluoride output.

Several studies (Bashash et al., 2017; Green et al., 2019; Lin et al., 2023) have used spot urine samples, either unadjusted or adjusted for creatinine or specific gravity, to assess non-dental health outcomes of systemic fluoride exposure at the individual level. Some studies (Bashash et al., 2017; Green et al., 2019) reported consistent associations between urinary fluoride (whether unadjusted or adjusted for creatinine or specific gravity) and child neurodevelopment, whereas other research has not reported significant associations with IQ (e.g. Lin et al., 2023). These findings highlight that variability in spot urine metrics, timing of collection, and differences in fluoridation modality can introduce uncertainty when linking individual urinary fluoride to health outcomes. This underscores the need for a structured and standardised approach to urine collection. We therefore recommend that future studies examining the impact of fluoride on health outcomes implement tailored urine collection protocols that account for: (i) the specific context, including age, location, and additional sources of fluoride; (ii) the fluoridation modality (i.e. naturally occurring or artificially added), as this may influence the stability and variability of exposure over time across a population, rather than biological effects; and (iii) the type of monitoring or research being conducted, whether at the individual or community level.

Environmental and physiological factors (such as altitude, diet, and age or growth rate) should also be considered when interpreting urinary fluoride concentrations, as they can substantially influence measured values. Collectively, these considerations highlight that while spot urine sampling offers practical advantages for population-level monitoring, its use requires careful methodological planning. Accounting for these factors when designing sampling schedules and interpreting results can improve the accuracy of population fluoride assessments and inform recommendations and guidance for optimal timing and sample collection strategies.

In conclusion, this multi-centre study confirms that 24-h urinary fluoride excretion remains the most reliable measure of fluoride exposure in children. Spot urine measurements, particularly U_{FC}, can serve as a practical alternative for population-level monitoring, but their accuracy is influenced by the timing of collection and the specific fluoridation modality. The variability in fluoride excretion seen across different exposure sources highlights the need for standardised approaches and protocols for monitoring systemic fluoride exposure in diverse paediatric populations.

4.1. Strengths and limitations

This study presents several notable strengths. First, validation of analytical methods across multiple laboratories enhanced the reliability and comparability of fluoride measurements. Second, the inclusion of a diverse sample of 178 children across varied geographic regions and fluoridation modalities, including community water, milk, salt fluoridation, and non-fluoridated settings, allowed for a broad assessment of systemic fluoride exposure. The sample size was adequate and aligned with WHO recommendations, and a standardised protocol for urine collection and analysis (including both 24-h and timed spot samples) strengthened the study's methodological rigor and reproducibility. Together, these features provide a robust and comprehensive evaluation of urinary fluoride excretion patterns in children.

However, several limitations should be acknowledged. The study focused on a narrow age range (4–6 years), which may limit generalisability to other paediatric age groups, especially given age-related differences in fluoride metabolism and retention. While major fluoridation modalities were represented, other sources of fluoride exposure (such as diet, use of fluoride-containing dentifrices, and environmental factors) were not fully accounted for and may have contributed to individual variability. In particular, the fluoride concentration of the toothpastes used by children was not directly measured. Given the study's focus on urinary fluoride biomarkers, the generally regulated nature of toothpaste fluoride levels, and the fact that intake from toothpaste is largely determined by brushing and ingestion behaviour, label-declared concentrations were considered sufficient for the purposes of this study. Sex was recorded, but the distribution of boys and girls was unequal within each site/modality, and the study was not specifically powered to examine sex differences. While including child sex as a covariate could theoretically confirm the robustness of our findings, some site-specific subgroups had relatively small sample sizes, which may limit the reliability of covariate-adjusted models. Weight-standardization of urinary fluoride per kg body weight provides partial normalization for sex-related physiological differences. Information on race/ethnicity was not collected; while some studies (Khan et al., 2025) suggest urinary fluoride levels may vary by sex or race/ethnicity, these differences likely reflect variability in exposure rather than established differences in fluoride metabolism.

Sociodemographic diversity across regions may have contributed to variability in creatinine- and specific gravity–adjusted urinary fluoride measures and their predictive ability for 24-h urinary fluoride, and associations might differ in more homogeneous populations.

CRediT authorship contribution statement

Fatemeh Vida Zohoori: Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **Marilia Afonso Rabelo Buzalaf:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Anne Maguire:** Writing – review & editing, Methodology, Conceptualization. **Roy Sanderson:** Writing – review & editing, Methodology,

Formal analysis. **Rodrigo A. Giacaman:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Stefania Martignon:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Edgar O. Beltran:** Writing – review & editing, Validation, Supervision, Data curation. **Yaysa Vasquez:** Writing – review & editing, Data curation. **Fatemeh Eskandari:** Writing – review & editing, Data curation. **Jelena Kronic:** Writing – review & editing, Validation, Formal analysis. **Karla Gambetta-Tessini:** Writing – review & editing, Supervision, Data curation. **Flavia Mauad Levy:** Writing – review & editing, Data curation.

Statements of ethics

All procedures were performed in compliance with relevant laws and institutional guidelines and have been approved by the appropriate institutional committees, including Teesside University, School of Health and Life Sciences Research Ethics Committee (#007/19, Oct/2019) in the UK, the Ethics Committee of Sao Paulo University for the study site in Bauru/Brazil (# CAAE 12565319.9.0000.5417, Jul 2019), the Ethics Committee of the University of Talca (#05-2020E, Sept/2023) in Chile and the Administrative and Research Board of the Universidad El Bosque (UEB-558, Sep/2020) in Colombia. The privacy rights of human subjects have been observed and written informed consent was obtained from the parent/legal guardian of the children recruited, prior to the study.

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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Conflict of interest statement

All authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijheh.2026.114795.

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