‘Assessment of fluoride exposure by GCMS: successes achieved and hard lessons learnt’

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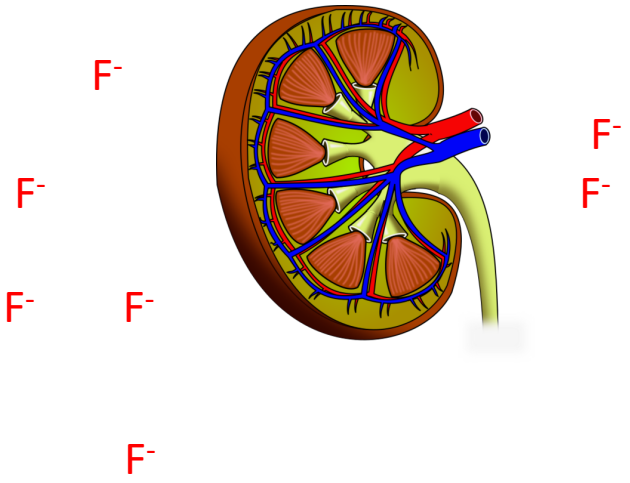
European Bioanalysis Forum

13th Open Symposium

November 2020

Introduction: why a fluoride assay

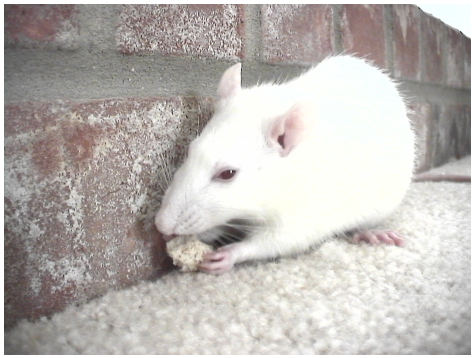
There are several volatile anaesthetics containing fluorine
e.g. sevoflurane, methoxyflurane, isoflurane.
Issues with nephrotoxicity of fluoride based anaesthetics were first reported in the 1960s
A result of increased fluoride exposure following metabolism
e.g. demethylation of methoxyflurane following cyp P450 oxidative metabolism



Since then every new volatile F based anaesthetic has been extensively tested for its potential to undergo metabolism, increase plasma F concentrations and thereby induce nephrotoxicity

Fluoride toxicity: background

Studies with rodents showed changes in renal function
could occur following intravenous dosage of inorganic Fluoride



When peak F concentrations reach: <40µM (760ng/mL): no abnormalities observed
50–80µM (1 to 1.6µg/mL): sub clinical toxicity observed
mild toxicity observed between 90-120µM and overt nephrotoxicity with
peak fluoride was observed at concentrations 80 - 175µM (1.6 to 3.3 µg/mL).
While this is does not give the whole picture of anaesthetic fluoride toxicity,
Need an analytical method capable of measuring plasma free F at 10µM or lower

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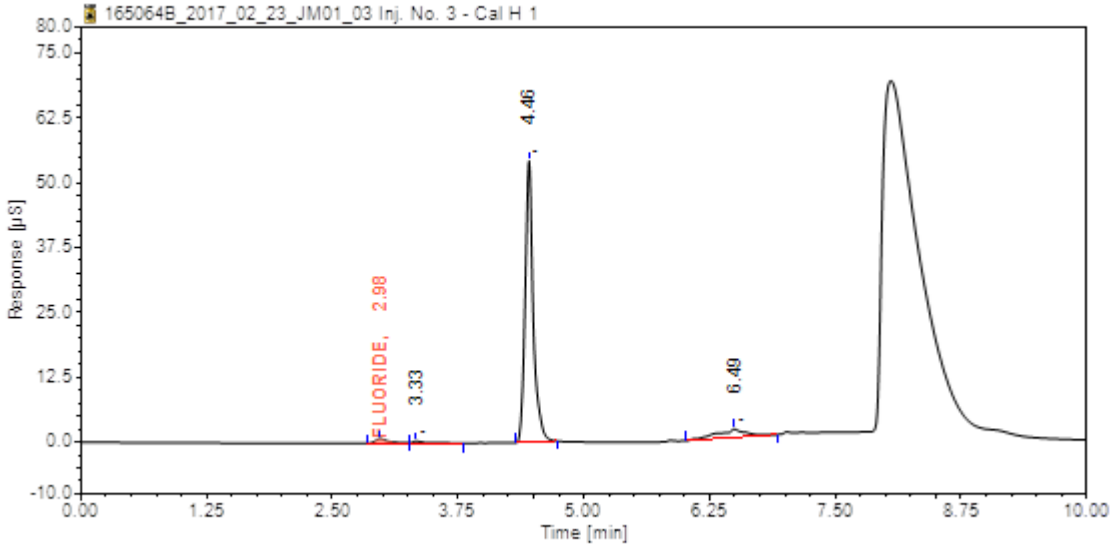
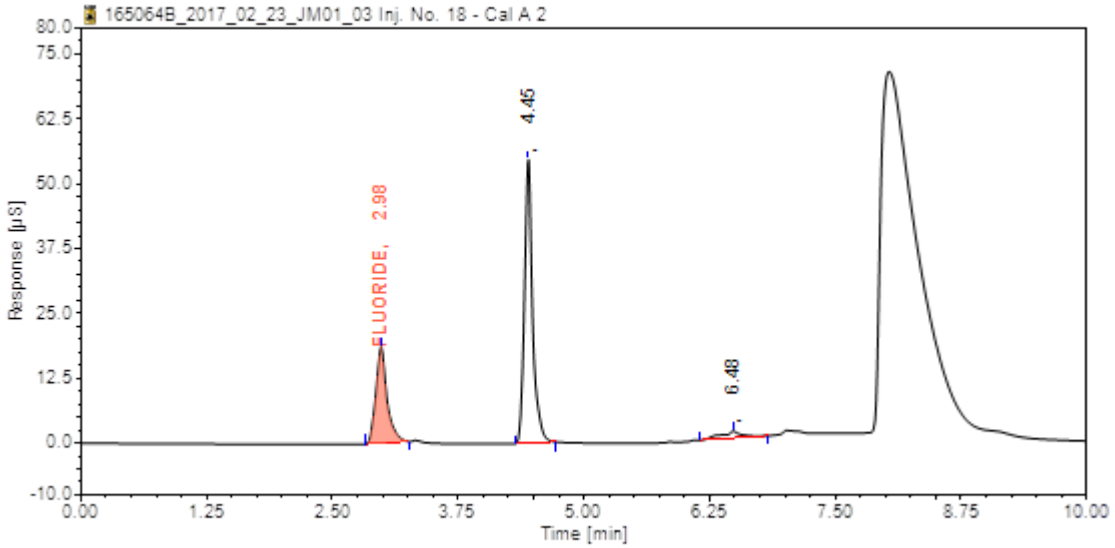
Free fluoride – analytical methods

Initial testing on site using ion chromatography (Dionex ICS)



Cal H – 1µg/mL
Limit of
Quantification



Cal A – 20µg/mL
Upper limit tested

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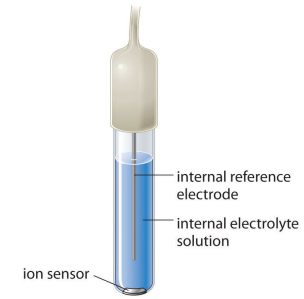
Fluoride –endogenous levels and detection limits

- LOQ of 1µg/mL with IC with a long incubation time and run time – undesirable for a clinical trial with large sample numbers
- Toxicity of fluoride observed at 50µM (~1µg/mL), the LOQ needs to be reduced to ensure a margin for patient safety
- Endogenous free fluoride levels in plasma vary according to diet, fluorination, ingestion of F containing dental products etc.

  literature values around 17ng/mL in plasma

(Sener et al., 2007, measured using an ion sensitive electrode)



Ion-selective electrode

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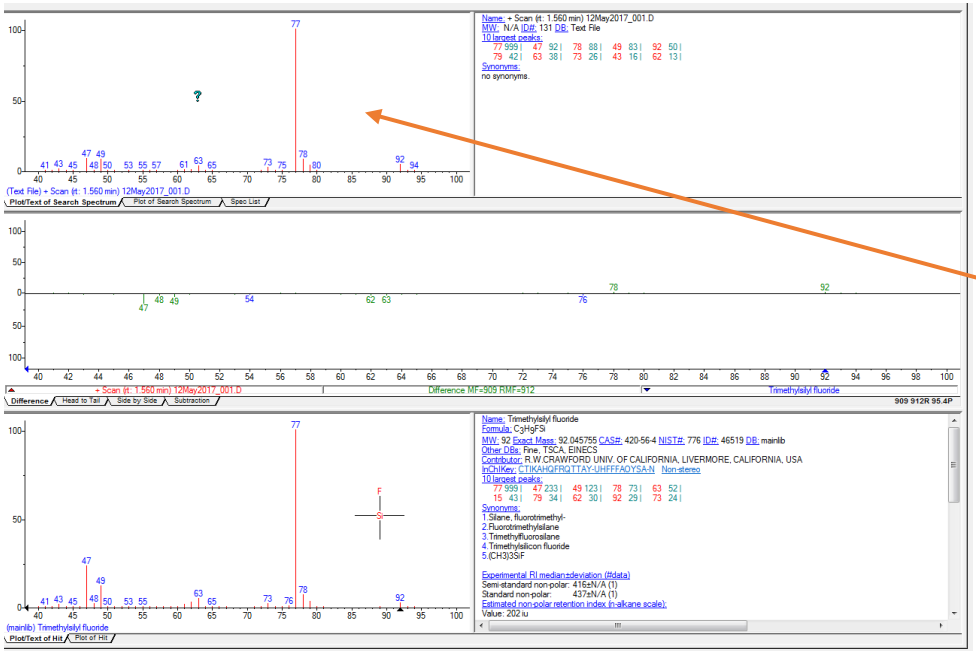
Chromatographic method for fluoride

Possible method based on that of Tsuda et al., 2016

Derivatisation of free fluoride to trimethylfluorosilane using TMCS (trimethyl chlorosilane) as a derivatising agent



EI mass spectrum of derivatised F vs NIST library spectrum

Molecular ion at m/z 92 (TMFS)
Major fragment at 77 (loss of CH_3)

Selected 77 as quantifier ion
92 as qualifier ion

Use acetaldehyde as an internal standard
Quantifier m/z 44
Qualifier m/z 29

$(CH_3)_3SiCl + H_2O \rightarrow (CH_3)_3SiOH + HCl$

$(CH_3)_3SiOH + H_+ + F_- \rightarrow (CH_3)_3SiF + H_2O$

(reaction equation from Tsuda et al., 2016)

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Sample preparation for plasma (Tsuda method was headspace in urine)

Free fluoride being analysed as a marker for **potential nephrotoxicity**
Endogenous assay using a calibration line in **surrogate matrix** (water)
Quality control samples prepared in **sample matrix** (human plasma)

2 major steps:

1) *delipidation and deproteination*

dilute sample with water and acidify
add dichloromethane & vortex
remove aqueous supernatant

2) *Fluoride derivitisation and extraction*

addition of internal standard in solvent
add TMCS in dichloromethane
incubate to derivatise; spin and transfer lower layer to a GC vial

Plasma volume for clinical study 1mL
Preclinical study 0.5 mL

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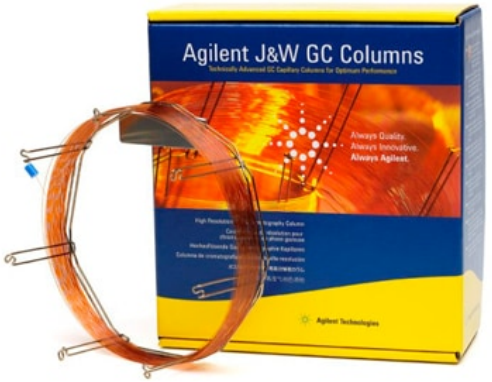
GCMS System

Agilent 5977 MSD Detector extractor EI source with 7890 GC oven

Heated inlet , split injection 5:1



Agilent DB Wax UI GC column
30 x 0.25mm 0.5µm film thickness

PEG based stationary phase for improved retention of polar compounds

Photo: Agilent

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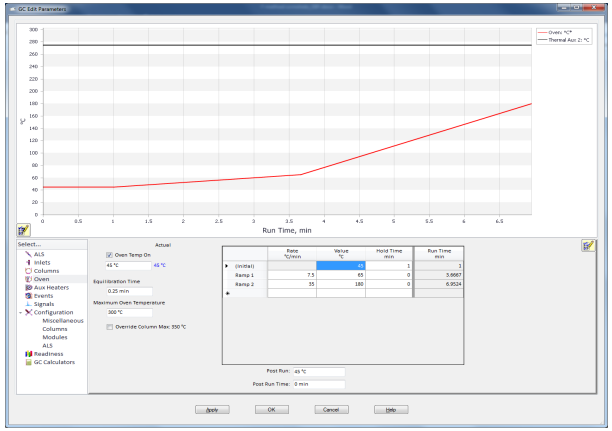
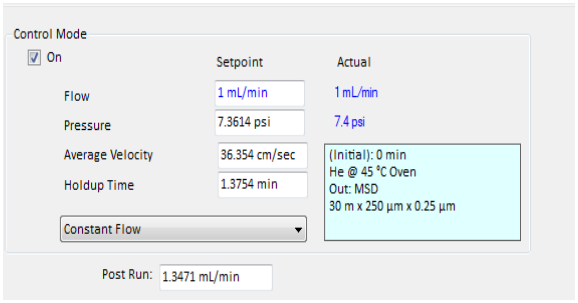
Analytical conditions





Gerstel MPS Autosampler

10uL syringe, mode;

sandwich with solvent above

Injection volume 1.5uL

2 washes: methanol and methanol/DCM 1:1

Total cycle time 7.5 minutes

Heated Inlet 200°C
Split mode
Helium flow 1mL/minute

45°C 1 minute
45 - 65°C at 7.5 °C/minute
65 - 180°C at 35°C/minute

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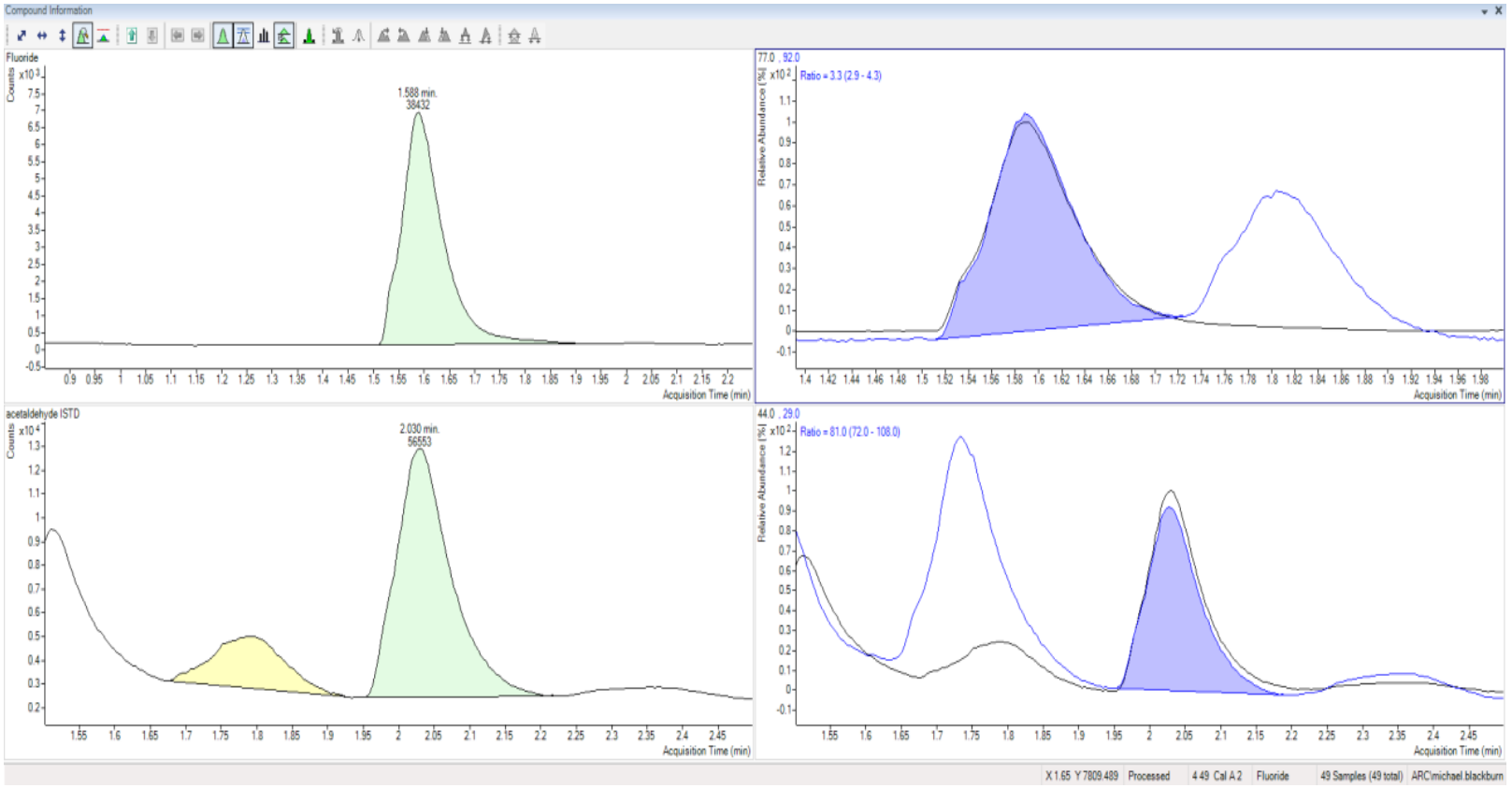
GCMS chromatograms showing qualifier ions



Ratio of fluoride quantifier to qualifier should be between 2.9 - 4.3: 1

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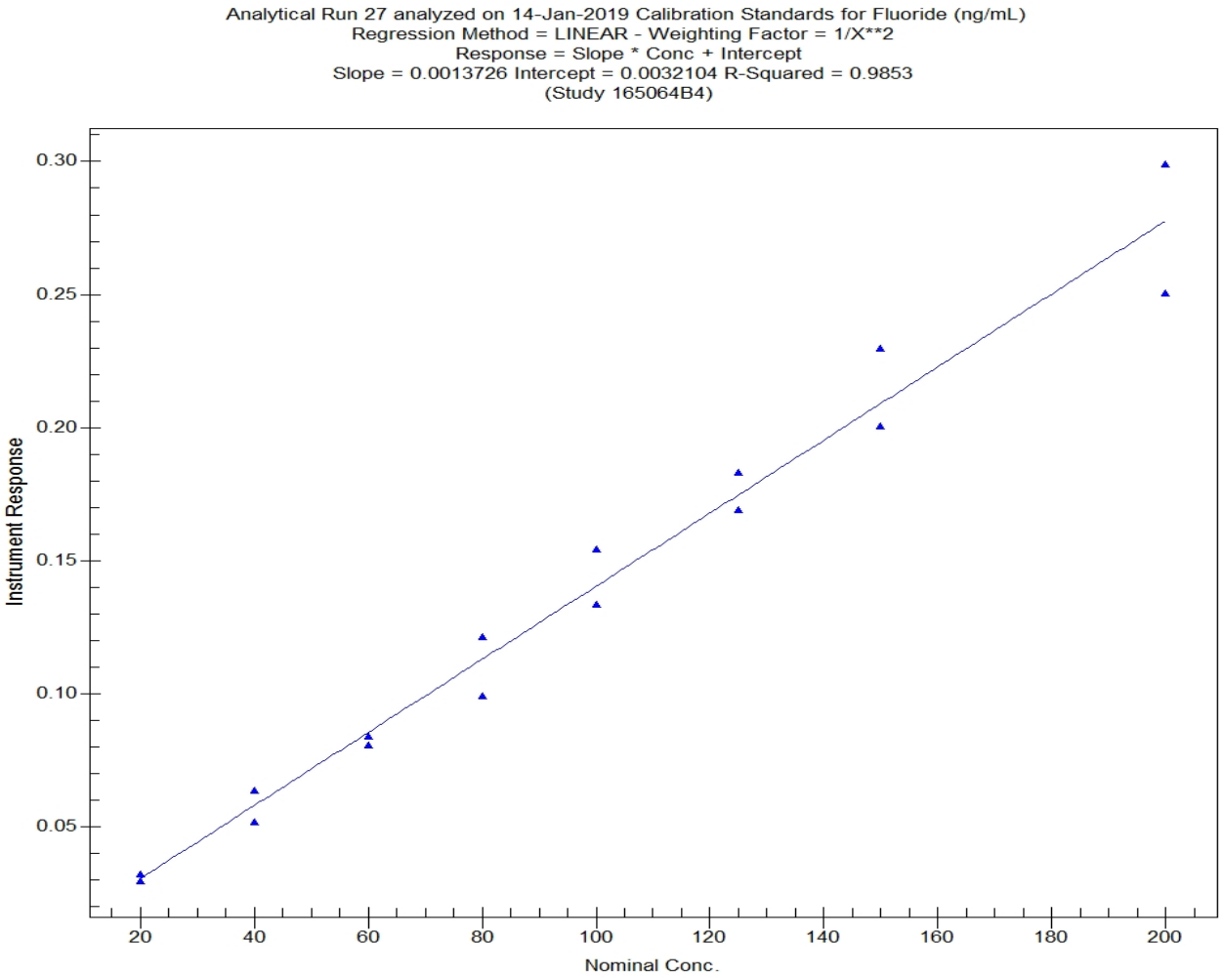
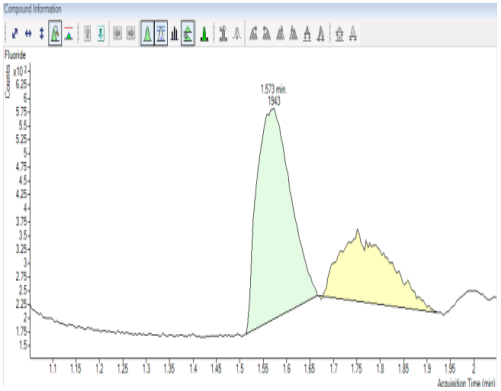
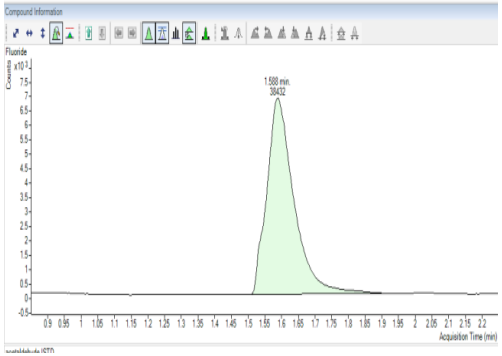
Validated assay calibration curve





Limit of quantitation



Higher limit of quantitation

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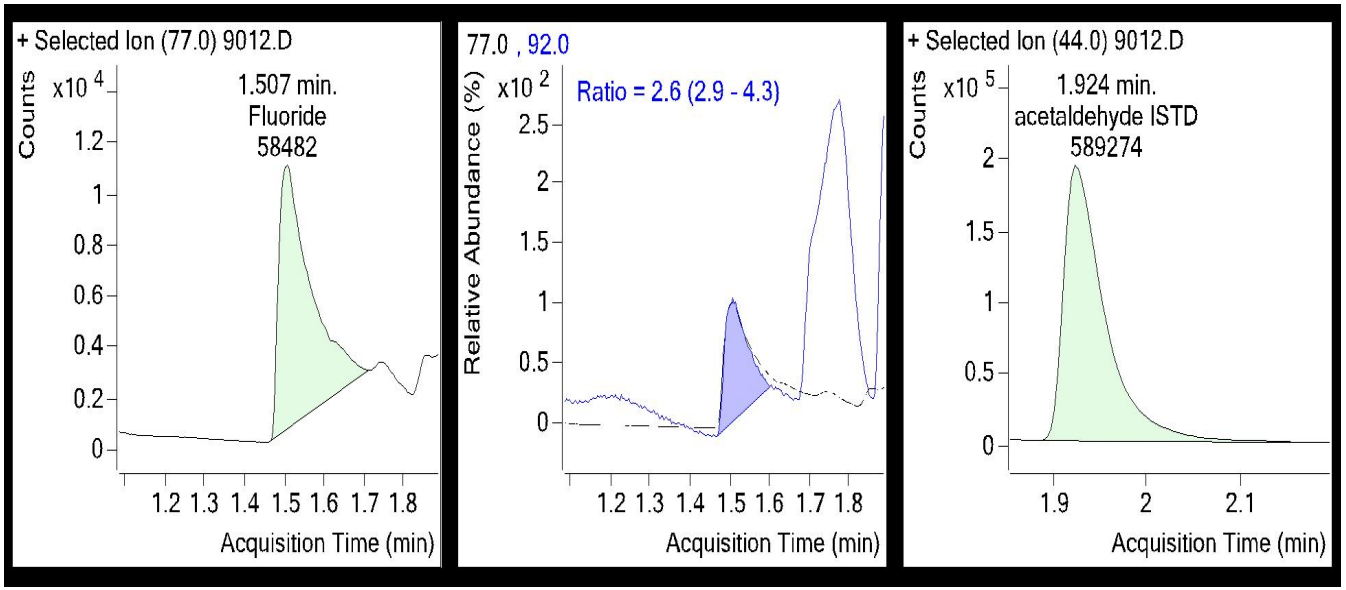
Blank plasma showing endogenous fluoride



GCMS LLOQ = 20ng/mL or approx. 1µM/mL
Endogenous values were generally at or a little below LLOQ: e.g. scan one values were extrapolated to 8-12 ng/mL

Blank plasma spiked to 4 nominal QC/Val levels at 25, 55, 105, 155 ng/mL

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Typical assay performance

	Curve Number	A&P LLOQ 26.0 ng/mL	A&P QCL 45.0 ng/mL	A&P QCM 102 ng/mL	A&P QCH 137 ng/mL
	9	~19.4	37.5	90.6	136
		21.7	39.1	95.2	126
		*184	~~20.7	~~19.4	~~201
		24.2	42.6	97.6	137
		28.5	43.7	97.4	140
		26.9	44.5	93.2	132
Mean		24.1	38.0	82.2	145
S.D.		3.71	8.91	30.9	27.7
%CV		15.39	23.45	37.59	19.10
%Theoretical		92.69	84.44	80.59	105.84
%Bias		-7.31	-15.56	-19.41	5.84
n		5	6	6	6
* Grubbs Test Outlier					
~ > 25%Bias					
~~ > 20%Bias					

- Acceptance criteria for this assay were expanded at 4/6/20-25% due to the unusual nature of the assay and consistent with data obtained during method development and pre-validation
- Analytical range truncated as accuracy dropped >200ng/mL (saturation of derivatisation = underestimation of nominal values?) Could not increase TMCS level – contains F

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Effect of dilution & non-parallel results

Method was validated into production.
During validation dilution was verified using plasma QCs spiked with fluoride standard
Samples could be diluted 2 & 5 fold with water

However similar dilution of INCURRED samples > ULOQ gave a non linear response
Samples diluted with water gave an overestimate of original concentration

Dilution 

Overestimate 

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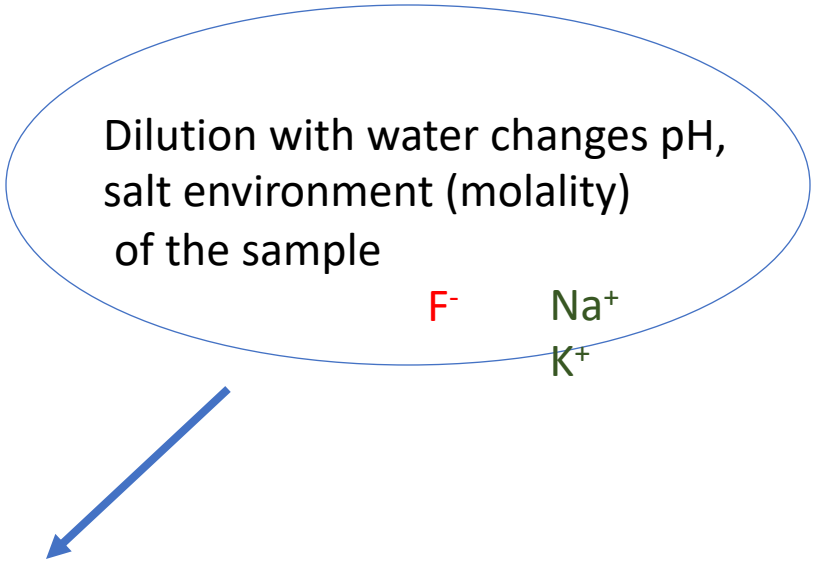

Release of free fluoride into the sample F^- from bound form in fluorinated organics or increased efficiency of derivatisation process?

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Non parallel assay: dilution effect

Dilution 

Solution: dilute sample with plasma matrix & not water to maintain molality, environment of sample

Overestimate 

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Non parallel assay: dilution effect

Dilution 

Solution: dilute sample with plasma matrix & not water to maintain molality, environment of sample

Overestimate 

However dilution plasma contains fluoride. Need to Assay dilution plasma for F first (or spike if <LOQ) & correct the measured diluted value for the additional spiked plasma

e.g. 2 fold dilution of plasma with plasma of nominal 28ng/mL
Results in a correction of the diluted value from 240 to 210 ng/mL

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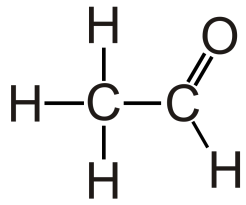
Internal standard effect

Attempted to find an internal standard which could be derivatised

We tried Br^- but this did not behave like F^- (did not derivatise)

Selected acetaldehyde (used by Tsuda et al).



This is not derivatised and does not track the analyte through the method
Lost extraction of acetaldehyde during production sample analysis

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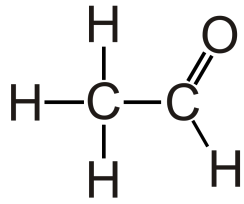

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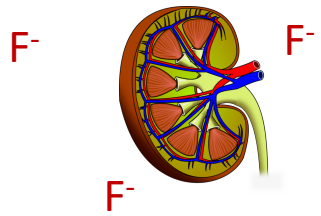
SOLUTION

Had to modify solvent in which the IS was added from methanol to dichloromethane and subsequently retest the assay
The reason for this was mysterious:
dichloromethane crossed the thin emulsion layer more easily?

conclusions



- Development of a volatile F containing compound required a fluoride assay as well as PK assay for drug to monitor potential for nephrotoxicity

- GCMS of a TMCS derivative allowed quantitation of free fluoride to clinically relevant concentrations

- The assay had a limited analytical range (saturation of TMCS?) and used a calibration curve in surrogate and spiked matrix QCs

- When diluting samples, consider the effect of the diluent on the sample, particularly for ionic analytes and derivatisation reactions

- Dilution of incurred samples had to be modified

- Select internal standard with care to track your procedure as far as possible

And expect the unexpected

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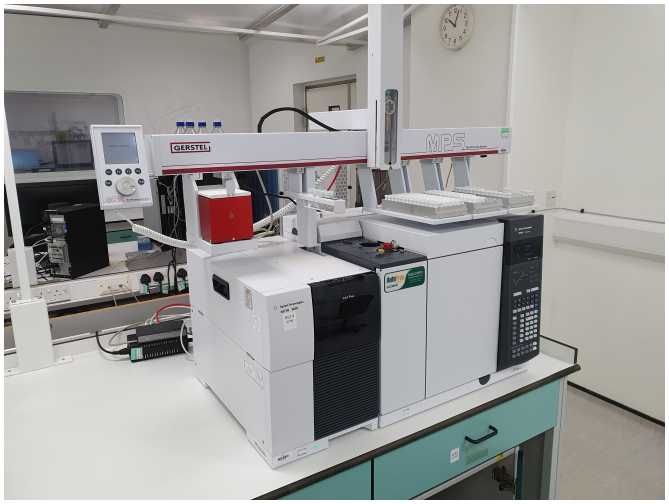
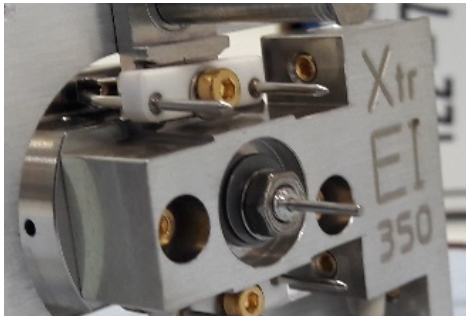
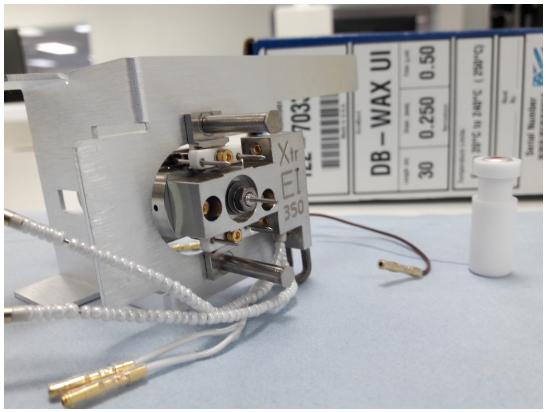
acknowledgements

Janine Morsman
Sheryl Atkinson
Stuart McDougall
Paige Bellis
Hannah Gent
Jonathan Charlton







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references

Sener et al., (2007) Fluoride levels of human plasma and breast milk *European Journal of Dentistry Vol. 1 pp 21-23*

Kharasch E.d. et al., (2006) New insights into the mechanism of methoxyfluorane Nephrotoxicity and implications for anaesthetic development. *Anaesthesiology 105 pp. 726 -736*

Tsuda Y et al., (2016) Measurement of fluoride in human urine by headspace Gas chromatography-mass spectrometry. *Biomedical Research on Trace Elements 27 (1) pp. 15-22*

Thank you for your attention