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Capillary gap flow cell as capillary-end electrochemical detector in flow-based analysis

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Graphical abstract

Highlights

- Gap flow cell (gap-FC) characterised and demonstrated as a capillary-end electrochemical detector.

- Gap-FC capillary outlet-electrode gap distance of just 30 μm .
- Gap-FC characterised for current, efficiency and effective cell volume.
- Highest efficiency 11% and lowest effective cell volume 35 nL achieved at 30 μm gap distance.
- Analytical performances compared with UV detection in capillary and standard LC.
- Gap-FC achieved lowest LODs of ascorbic acid, 2,3-dihydroxybenzoic acid and pyrocatechol to date.

Abstract

A gap flow cell (gap-FC) as a capillary-end electrochemical (EC) detector, incorporating exchangeable screen-printed electrodes, that facilitates adjustment of the gap distance between the capillary outlet and working electrode down to 30 μm was investigated. The analytical performance of the gap-FC was assessed in terms of detector response (current), the efficiency of EC conversion of ferrocyanide, and effective cell volume, as a function of flow rate and gap distance, within a range of 0.1-200 $\mu\text{L min}^{-1}$ and 30-100 μm , respectively. As a result, an efficiency of EC conversion within the range of ~1.5-11% (~3-16 times higher than wall-jet FC), at an effective cell volume of ~30-75 nL, was achieved. The gap-FC was applied as a liquid chromatography EC detector, coupled with standard and capillary format liquid chromatographs, where the analytical performance of gap-FC was compared with that of a UV detector. With the standard LC, the sensitivity for ascorbic acid, 2,3-dihydroxybenzoic acid and pyrocatechol, was greater than with the UV detector, with limits of detection (LODs) one order of magnitude lower for the gap-FC (0.10 μM , 0.09 μM , 0.19 μM , respectively, RSD 1.35-3.8 %, $n=9$, linearity $r^2 \sim 0.99$ for concentration range 1-100 μM). LODs of test electroactive solutes using the gap-FC were ~2 times lower than LODs reported in existing literature using standard EC detectors, including wall-jet and thin-layer FCs.

Keywords

Conversion efficiency

Effective cell volume

Flow-based analysis

Gap distance

Gap flow cell

Abbreviations

A	Electrode geometric area
A_s	Peak asymmetry factor
2,3-DHBA	2,3-dihydroxybenzoic acid
ACN	Acetonitrile
AD	Amperometric detection
Ag	Silver
C	Concentration [mol L^{-1}]
C^4D	Capacitively-coupled contactless conductivity detector
dN/dt	Rate of change of number of moles
EC	Electrochemical
F	Faraday constant [C mol^{-1}]
FC	Flow cell
$[\text{Fe}(\text{CN})_6]^{4-}$	Ferrocyanide
FIA	Flow injection analysis
FWHM	Full width at half maximum
HPLC	High-performance liquid chromatography
I_0	Theoretical current corresponding to full conversion [A]
I	Measured current [A]
LC	Liquid chromatography
LOD	Limit of detection

η	EC conversion efficiency [%]
n	Number of electrons transferred per mole of solute
N	Number of moles of solute oxidised or reduced
N_p	Plate number
NaOH	Sodium hydroxide
PEEK	Polyether ether ketone
Pt	Platinum
Q	Flow rate [L min^{-1}]
r	Correlation coefficient
t_R	Retention time
RE	Reference electrode
RP	Reversed-phase
S/N	Signal-to-noise ratio
SPEs	Screen-printed electrodes
t	Time [min]
TL-FC	Thin-layer flow cell
V_C	Volume of capillary
V_D	Volume of the detector (gap-FC)
V_E	Effective cell volume
V_I	Void volume contribution by the injector
$w_{h/2}$	Width of the peak at half height
w_R	Distance from peak midpoint to trailing edge of the peak
w_L	Distance from leading edge of the peak to peak midpoint

WJ-FC	Wall-jet flow cell
WE	Working electrode

1. Introduction

The prospect of low-cost and miniaturised detection has led to the use of electrochemical (EC) detectors in flow-based analytical systems such as flow injection analysis (FIA), capillary electrophoresis, and liquid chromatography (LC) [1-6]. However, incorporating EC detectors in flow-based systems is challenging, specifically when attempting the physical and chemical characterisation of the EC detector. Wang [5] discussed the design, principles and applications of EC detectors for microfluidic-based systems. Xu et al. [6] reported a novel microelectrode design, fabrication methods, versatility of EC techniques for identifying of biochemical processes, and applications such as in situ analysis, point-of-care testing and portable analysers for complex sample matrices. The most commonly used EC detector is thin-layer flow cell (TL-FC). In TL-FC, the solution flows through a thin flat channel parallel to the working electrode (WE) surface [7-9]. In another type of commonly used EC detector is wall-jet FC (WJ-FC) [10], where the solution stream is directed perpendicularly to the plane of WE [11] through a small orifice and forms a jet that impinges on the electrode surface [12].

There exist a number of significant drawbacks and research challenges associated with the existing TL- and WJ-FC as EC detectors. These include difficulties in miniaturisation of the system [13-15], frequent repolishing of the electrode [16], and low efficiency of EC conversion, typically 4-5% [17]. Additionally, there are technical issues such as a lack of experimentally determined effective cell volume (V_E) data for the different gap distance (capillary outlet-electrode) in existing EC detectors [18-21], as well as the necessity of cleaning the electrode surface during or after the experiment, and lack of EC detectors capable of being coupled with chromatographic separation platforms operating at flow rates within the nano- to millilitre per minute range [13, 16, 17, 19-22]. The existing mechanisms of TL- and WJ-FC in terms of layer and jet formation is reported in Hubbard *et al.* [23], Gunasingham [13], and Patthy *et al.* [16] respectively, which stipulate rigid construction of the EC detection FCs. In flow-based analytical systems, variations in the rigid construction of TL- and WJ-FC such as adjustment of effective cell volume, flow rates as well as the incorporation of miniaturised electrodes made of different materials are required.

Recently, BVT Technologies has designed a new EC detector (termed gap-FC in this study), which enables the use of exchangeable electrodes in a flow-through arrangement. The inherent features of this new gap-FC for EC detection

overcome some of the above-mentioned difficulties associated with EC detectors in flow-based systems. Unlike TL- and WJ-FC, the gap-FC enables simple adjustment of the gap distance between the surface of the WE and the capillary orifice, down to a few microns, to facilitate the formation of a very thin layer of electrolyte on the electrode surface. At very low flow rates and small gap distances, the gap-FC can be used to precisely investigate current dependencies, efficiency % of EC conversion, and the exact V_E . As the gap-FC also incorporates exchangeable electrodes, specifically screen-printed electrodes (SPEs), the choice of potential WE materials is extensive. SPEs are typically inexpensive, simple, reliable to operate with high selectivity and precision [24], and offer a quick response and high sensitivity [25]. Additionally, the new gap-FC is small in size, robust, and relatively low cost (<\$800), which makes it an ideal solution for portable and miniaturised analytical systems.

Comparing the design of gap-FC with TL- and WJ-FC designs, we emphasize that gap-FC is not similar to either TL- or WJ-FC. In TL-FC, the flow direction is horizontal to electrode surface whereas, in WJ-FC the flow direction is vertical to the electrode surface. Recently, we have published a review paper [1], where the flow profiles in EC FCs were schematically illustrated. Compared to TL- and WJ-FC, gap-FC enables adjustment of the gap distance between the surface of the WE and the capillary orifice, down to a few microns, to facilitate the formation of a very thin layer of electrolyte on the electrode surface which resulted the effective cell volume of the gap FC. One can expect all the advantages of thin layer FC in gap-FC with the added advantage of controlling the effective cell volume. According to literature, the WJ-FC were constructed with minimum gap distances in 0.125-10 mm range and minimum flow rate in 0.7-19.6 mL min⁻¹ range [11, 13]. The construction of gap-FC accommodates very low flow rates (0.1-200 μ L min⁻¹ in this study) and minimal gap distance (30-100 μ m in this study). We emphasize that in our study of the gap-FC, a layer is formed inside at such low flow rates and gap distance, for which we demonstrated the accurate measurement of the resulting effective cell volume inside the gap-FC. We acknowledge that at high flow rates in mL range and at larger gap distance in mm range, the gap-FC may act as WJ-FC, however, these configurations were deemed unsuitable for miniaturised capillary platforms in this study.

There is a significant number of publications covering the topic FIA coupled with SPEs, where most of these works illustrated the application of the techniques [20, 21, 26-29]. Additionally, researchers employed biologically modified electrodes in micro FCs for AD in flow mode [26-29], however without demonstrating their effective coupling with separation platforms, mainly due to lack of characterisation of these EC biosensors in terms of detector response (current), the efficiency of EC conversion, and V_E , at a range of flow rates and gap distances. We

emphasize the fact that a detailed performance analysis of these EC detectors [20, 21] is the prerequisite for their coupling with separation platforms [26-29]. To date, studies on detailed performance analysis of miniaturised EC detectors that could be effectively coupled with FIA, capillary and standard LC systems are limited in their number and scope.

The aim of this study was to evaluate the performance of miniaturised EC detector (i.e., gap-FC) in EC detection for the first time and then demonstrate its application within the performance range when coupled with flow-based systems, specifically FIA, capillary and standard LC. Therefore, within this work, we investigated the analytical performance of the gap-FC incorporating SPEs using a simple FIA set-up, in terms of detector response (current), the efficiency of EC conversion, and V_E , at a range of flow rates and gap distances. Having the performance range of the gap-FC characterised, we then investigated the analytical application of the gap-FC in capillary and standard LC. SPEs were reported as in-house/commercially available exchangeable electrodes used in EC sensors/biosensors [26, 34], and hence, in our study we employed commercially available SPEs as exchangeable electrodes in gap FC. The electroactive species, namely, ascorbic acid, 2,3-dihydroxybenzoic acid, pyrocatechol and dopamine were used as test solutes in this study. These solutes were previously reported in different flow-based analysis systems using EC detectors and therefore allowed direct comparison with previous works [15, 30-32]. The analytical application of the gap-FC in LC was also investigated, comparing the detection sensitivity of the gap-FC with EC detectors reported in the literature, as well as with a standard UV detector.

2. Experimental

2.1 Chemicals

Analytical grade standards and reagents were used in this study. These were potassium ferrocyanide trihydrate ($K_4Fe(CN)_6 \cdot 3H_2O$, Ajax, Australia), sodium hydroxide (NaOH, Sigma-Aldrich, Sweden), dopamine, ascorbic acid, 2,3-dihydroxybenzoic acid (2,3-DHBA), pyrocatechol, citric acid, sodium citrate dihydrate (Sigma-Aldrich, USA), acetonitrile (ACN, 99.8% HPLC grade, VWR, Australia), and milli-Q water (Millipore, USA). HPLC grade solvents were used for all separations. All test solutes and mixtures were freshly prepared in the same solvent such as ACN and citrate buffer used for the LC mobile phase.

2.2 Instrumentation

A simple micro-flow injection analysis (μ FIA) platform was assembled using a LabSmith uProcess™ microfluidic system (LabSmith, USA). The platform consisted of two programmable microsyringe pumps (LabSmith model: SPS01) with 100 μ L glass syringes, one four port valve ('L' pattern flow, and 2 positions switching valve, LabSmith model: AV201-C360) and one injector valve (LabSmith model: AV303-C360). All pumps and switching valves were connected via PEEK tubing (Polyether ether ketone, 150 μ m i.d., 360 μ m o.d.) on a LabSmith uProcess™ Breadboard. LabSmith uProcess™ software was used for controlling the various components and the volumetric flow rate of microsyringe pumps [33]. The gap-FC was investigated using a polyimide coated fused silica capillary (100 μ m i.d., 360 μ m o.d., length 7-12 cm, TSP100375, Polymicro Technologies™, USA) to connect the injector valve and the gap-FC. The gap-FC itself was a three-electrode FC configuration, of dimensions - length 30 x width 16 x height 17 ± 0.05 mm, and model FC5 in PEEK (BVT Technologies, Czech Republic; see Fig. 1, ESI Fig. S1, Fig. S2, and section S1). A TraceDec® capacitively-coupled contactless conductivity detector (C^4D , Innovative Sensor Technologies GmbH, Austria) was incorporated into the set-up to allow peak area comparisons for V_E calculations. The full instrumental scheme is shown in ESI (see Fig. S3).

Fig. 1. Gap-FC with SPEs (plan view and a cross-section of measurement part, where D1: working electrode 2.05 mm, D2: reference electrode 4.60 mm, D3: auxiliary electrode 5.95 mm, D4: dielectric layer 7.65 mm, D5: passage for capillary 0.36 mm, D6: hole for capillary 0.4 mm, D7: waste 0.3 mm, h: gap distance, O: O-ring, S: SPEs thickness 0.63 mm).

Both capillary and standard LC platforms were used in this study. The capillary LC (Dionex ultimate 3000 Nano HPLC, Thermo Scientific™, USA) consisted of a thermostated reversed-phase (RP) capillary column (SGE ProteCol C18 G 123 column, length 15 cm x i.d 150 μ m, particles size 3 μ m, 120 Å, Trajan Scientific and Medical, Australia), a multi-wavelength UV-Vis photometric detector (UltiMate™ 3000 VWD variable wavelength detector, path length: 10 mm,

cell volume: 45 nL, Dionex™ Thermo Scientific™, USA) and injector valve (MV303-C360, LabSmith, USA). The capillary LC platform was operated using Chromeleon 6.8 software (Thermo Scientific™, USA). The standard LC platform (Alliance Waters 2690 HPLC, Waters, USA) consisted of thermostated RP column (YMC Pac Pro C18, length 25 cm x i.d. 4.6 mm, pore size 5 µm, YMC Co. Ltd, Japan), UV detector (Waters 996 PDA detector, path length: 10 mm, cell volume: 8 µL, Waters, USA) and injector valve (SM4, Waters, USA). The standard LC was operated by the Empower software (Waters, USA). The gap-FC was coupled to both capillary and standard LCs.

AD was performed under optimised conditions, where +800 mV using a platinum (Pt) as WE, with a flow rate of 1 µL min⁻¹ in the capillary LC set-up, and 0.6 mL min⁻¹ with the standard LC, using a capillary i.d of 100 µm, and a gap distance of 30 µm. We have observed a higher UV signal (peak height) at 254 nm for ascorbic acid, and at 280 nm for 2,3-DHBA, pyrocatechol, and dopamine. Therefore, we used both 254 nm and 280 nm for UV detection in this study.

EC detection in both FIA and LC platforms was performed using the commercially available SPEs AC₁W₂R₅ as an exchangeable EC sensor of dimensions - length 25.40 x width 7.26 x height 0.63 ± 0.05 mm (BVT Technologies, Czech Republic). The electrode consisted of Pt as WE (diameter: 2 mm), silver (Ag) as a reference electrode (RE), and Pt as the auxiliary electrode. The potential at WE, which is measured versus the RE, is determined by the element metal of RE and the active species concentration in the electrolyte. In our study, we used screen-printed silver as a RE and the wet form of electrolyte ([Fe(CN)₆]⁴⁻ in NaOH solution). The form of the electrolyte, had no effect on the reference potential [34]. The SPEs materials and fabrication details are discussed in recent reviews by Li *et al.* [25] and Couto *et al.* [35]. The O-ring of the FC ensured no back mixing of the flowing solution (see video link in ESI section S1.1). A potentiostat (model ER466CE, eDAQ Pty Ltd, Australia) was connected to the gap-FC and operated with eDAQ software (eDAQ Pty Ltd, Australia) to perform EC detection.

2.3 Measurement of effective cell volume

In this study, the V_E of gap-FC was experimentally determined by varying the flow rate and gap distance. For a given set of conditions (such as flow rate, gap distance, and length of the capillary) simultaneous peak responses for the test solute [Fe(CN)₆]⁴⁻ were obtained using both on-capillary C⁴D and the gap-FC as EC detector, from a single injection. From the peak response (i.e., the height of the peaks), the total volume was calculated by multiplying the flow rate and time at half of the peak height [36] (see ESI Fig. S4). In general, for a certain flow rate and gap

distance, two sets of total volumes can be obtained from C⁴D and gap-FC by varying the length of the capillary. Detailed measurement and calculations of V_E are discussed in section 3.1.3 and ESI section S2.2.

3. Results and discussions

FIA was used to characterise the gap-FC in terms of detector response, the efficiency of EC conversion, and V_E . For experimental investigations, we used low concentration of $[\text{Fe}(\text{CN})_6]^{4-}$, which is highly electroactive as well as the most commonly used solute for the study of EC detection. In addition to EC detection in our study, we also incorporated C⁴D for gap-FC characterisation. The low concentration of $[\text{Fe}(\text{CN})_6]^{4-}$ was suitable to use in C⁴D platform in terms of detector sensitivity. Therefore, we have used 10 nM concentration of $[\text{Fe}(\text{CN})_6]^{4-}$. Observations of the baseline did not reveal any considerable fluctuations with the flow of the eluent in our study. This characterisation was undertaken prior to demonstrating the practical analytical application of the gap-FC in capillary and standard LCs.

3.1 Characterisation of gap-FC

3.1.1 Detector response (current)

The combined dependence of current on flow rate and gap distance for the gap-FC is shown in Fig. 2.

Fig. 2. Dependency of current on flow rate and gap distance, respectively (a. 3D graph and b. 2D graph). Conditions: solvent: water; flow rate: $0.1\text{--}200\ \mu\text{L}\ \text{min}^{-1}$; solute: $10\ \text{nM}\ [\text{Fe}(\text{CN})_6]^{4-}$ in $0.04\ \text{M}\ \text{NaOH}$; sample injection volume: $3\ \mu\text{L}$; capillary (injector-gap-FC): i.d $100\ \mu\text{m}$, length $7\ \text{cm}$; gap distances: $30\text{--}100\ \mu\text{m}$; and AD potential: $+650\ \text{mV}$.

The current in EC detector depends on the number of moles of solute that are oxidised or reduced at the WE. Obviously, flow velocity has a significant impact on the efficiency of this process. We observe in Fig. 2 that at low ($0\text{--}20\ \mu\text{L}\ \text{min}^{-1}$) and high ($>20\text{--}200\ \mu\text{L}\ \text{min}^{-1}$) flow rates, the signal always increased with a decrease in gap distance. At higher flow rates due to high sharper peaks with less dispersion in the fluidic path [37], detector response continued to increase but at a reduced rate. At flow rates above $75\text{--}100\ \mu\text{L}\ \text{min}^{-1}$, the response could be approximately doubled through reduction of the gap distance from 100 down to $30\ \mu\text{m}$. The rate of change of signal with flow rate below $20\ \mu\text{L}\ \text{min}^{-1}$ at a fixed gap distance of $30\text{--}100\ \mu\text{m}$ was appeared linear (see Fig. 2). The theoretical current in EC detectors can be expressed by equations 1 and 2 [17, 38].

$$I_0 = QCF \quad (1)$$

$$I_0 = nAF \frac{dN}{dt} \quad (2)$$

Where, I_0 = Theoretical current corresponding to full conversion at WE surface, Q = Flow rate [L min^{-1}], C = Concentration [mol L^{-1}], F = Faraday constant $96,485 \text{ [C mol}^{-1}\text{]}$, n = number of electrons transferred per mole of solute, A = Electrode geometric or projected area [m^2], N = Number of moles of solute oxidised or reduced, and t = time [min].

By combining equations 1 and 2, we can derive equation 3, where we can attribute the increased rate of change of the number of moles (dN/dt) at the electrode surface with the increased flow rate (Q), considering C and n as constants.

$$QC = nA \frac{dN}{dt} \quad (3)$$

Equations 1-2 doesn't contain any parameter related to the gap distance. Here higher currents were observed at smaller gap distances, although noise remained almost constant for all gap distances (see ESI section S3.1.4). Furthermore, we attribute the fact that the residence time of solute (i.e., $\text{K}_4\text{Fe}(\text{CN})_6 \cdot 3\text{H}_2\text{O}$) at the electrode surface reduces with decreasing gap distance. The increased responses from different analytes in terms of peak heights and widths at lower gap distances in EC detectors have also been observed and reported by Yamada *et al.* [11], Gunasingham [13], and Ji *et al.* [39]. Gunasingham [13] has previously shown this effect, noting that the short residence time caused increased peak current (signal) for some analyte (o-aminophenol, p-aminophenol and p-phenylenediamine), whereas decreased peak current for some other analyte (2,6-diisopropylphenol, 3-isopropylphenol and phenol). Gunasingham [13] attributed the alteration of the radial velocity profile of different analytes at the detection cell at a lower gap distance as a reason for this increase or decrease of peak current. Therefore, the analyte type is chosen in our study also played a role in the increased signal at lower gap distance. In this investigation, the dependencies are clearly demonstrating that current can be maximised at the lowest gap distance of $30 \mu\text{m}$ and can be further increased with the increase of flow rate at this lowest gap distance.

3.1.2 EC conversion efficiency

The dependence of EC conversion efficiency upon gap distance for solute $[\text{Fe}(\text{CN})_6]^{4-}$ was determined across the range of 30 to $100 \mu\text{m}$, with the resulting efficiency data shown in Fig. 3. Counter to the above overall signal enhancement with flow rate, the EC conversion efficiency observed to drop rapidly with the flow.

Fig. 3. The dependency of the efficiency of EC conversion % on flow rate and gap distance, respectively (a. 3D graph and b. 2D graph). Conditions: solvent: water; flow rate: 0.1-200 $\mu\text{L min}^{-1}$; solute: 10 nM $[\text{Fe}(\text{CN})_6]^{4-}$ in 0.04 M NaOH; sample injection volume: 3 μL ; capillary (injector-gap-FC): i.d 100 μm , length 7 cm; gap distances: 30-100 μm ; and AD potential: +650 mV.

The ratio of measured current I to the theoretical current I_0 corresponds to the EC conversion efficiency of the FC according to equation 4.

$$\eta = \frac{I}{I_0} = \frac{I}{QcF} \quad (4)$$

Where, η = EC conversion efficiency [%], I = Measured current [A].

The reduction of the EC conversion efficiency at the increased flow rate in Fig. 3 illustrates the inverse relationship between the efficiency of EC FCs with the flow rates described in equation 4 [17]. Krejci *et al.* [17] has previously shown this effect, noting that lower flow rates provide longer residence time and thus increased substrate conversion efficiency. Here, this effect is further eventuated with an increase in gap distance, as shown in Fig. 2 and Fig. 3. For the flow rates used in this study, EC conversion efficiencies ranged from ~ 1.5 % at 200 $\mu\text{L min}^{-1}$ to 11 % at

$0.10 \mu\text{L min}^{-1}$. However, the EC conversion efficiencies with the gap-FC were ~ 3 -16 times higher than those reported for a WJ-FC, for flow rates ranging from 1.5 to $200 \mu\text{L min}^{-1}$ [17]. Finally, the above characterisation studies confirmed that the minimum gap distance of $30 \mu\text{m}$ at the lowest flow rate of $0.10 \mu\text{L min}^{-1}$ showed maximum EC conversion efficiency for the gap-FC.

3.1.3 Effective cell volume

The V_E of the gap-FC varied across the range of 35 - 75 nL for different gap distances (30 - $80 \mu\text{m}$) and flow rates investigated in this study (see Fig. 4). Gunasingham [13] reported that small V_E reduce band spreading and enhance EC conversion efficiency. In this study, the maximum EC conversion efficiency of 11% was obtained at $30 \mu\text{m}$ gap distance and a flow rate of $0.5 \mu\text{L min}^{-1}$ (see Fig. 3). This relatively high EC conversion rate was achieved at an V_E of $\sim 35 \text{ nL}$, as illustrated in Fig. 4(a). In the past, the V_E of alternative EC detectors have been calculated based on applying a simplified method for a known detector geometry, by multiplying electrode surface area and gap distance [18, 19, 22]. In this present study, V_E has been measured experimentally, through varying gap distance and flow rate. The experimentally determined V_E of 35 nL for the gap-FC is the lowest amongst those reported V_E of EC detectors [18, 19, 22] and more importantly, the only experimentally confirmed V_E to-date, which produced an EC conversion efficiency of $\sim 11\%$.

Fig. 4. (a) Dependency of effective cell volume on flow rate and gap distance, respectively. Conditions: solvent: water; flow rate: 0.5 - $15 \mu\text{L min}^{-1}$; solute: $10 \text{ nM } [\text{Fe}(\text{CN})_6]^{4-}$ in 0.04 M NaOH ; sample injection volume: $3 \mu\text{L}$; capillary (injector-gap-FC): i.d $100 \mu\text{m}$, length 7 - 12 cm ; gap distances: 30 - $80 \mu\text{m}$; and AD potential: $+650 \text{ mV}$. (b) Calculated V_E for flow rate $5 \mu\text{L min}^{-1}$, and gap distance $50 \mu\text{m}$, which plotted as a single point in the 3D graph.

3.2 Analytical applications

In this study, to demonstrate compatibility across a wide range of flow rates, the analytical applications of the gap-FC were investigated in combination with both capillary LC, and standard LC systems (the latter with simultaneous UV absorbance detection). The separations were developed under isocratic conditions, and the system parameters, such as mobile phase flow rate, column to detector capillary i.d, gap distance, and applied potential, were optimised using chromatographic peak signal-to-noise ratio (S/N, see ESI section S3). Under optimised conditions calibration curves for test solutes were constructed for each LC format.

3.2.1 Standard LC-UV-EC

The chromatograms of target solutes using standard LC-UV-EC system are illustrated in Fig. 5.

Fig. 5. Chromatograms showing separations of ascorbic acid, 2,3-DHBA, and pyrocatechol using standard platform. Conditions: mobile phase (v/v=60:20:20): ACN: water:25 mM citrate buffer, pH 3.5; elution: isocratic; flow rate: 0.6 mL min^{-1} ; sample injection volume: $10 \mu\text{L}$; LC column (injector-UV detector): YMC Pac Pro C18 RP column (length: 25 cm, i.d: 4.6 mm, particles size: $5 \mu\text{m}$), column oven temperature: 25°C ; pressure: $\sim 1050 \text{ psi}$ ($\sim 73 \text{ bar}$); capillary (UV-gap-FC) i.d: $100 \mu\text{m}$, length: 7 cm; gap distance: $30 \mu\text{m}$; WE: Pt; and AD applied potential: +800 mV. UV absorbance wavelength: 280 nm. See 254 nm UV absorbance graph in ESI (Fig. S13).

Table 1. Comparison of analytical performance of gap-FC with UV detector in standard LC platform.

Detector	Analyte	Void Volume ^a [mL]	Peak asymmetry factor ^b	Peak efficiency (plate number ^c / column length) [N m ⁻¹]	Peak FWHM [min]	Baseline noise (3SD) 5-10 min	LOD ^d [μM]	RSD [%]
UV	Ascorbic acid	0.1	1.1-1.3	39800-62000	0.12-0.16	0.22 [mAU]	0.37	1.1-
	2,3-DHBA					(254 nm)	0.83	4.8
	Pyrocatechol					0.15 [mAU] (280 nm)	4.90	
Gap-FC	Ascorbic acid	-	1.0-1.2	36300-55400	0.10-0.13	1.5 [nA]	0.10	1.3-
	2,3-DHBA						0.09	4.9
	Pyrocatechol						0.19	

^a Void volume calculated by multiplying flow rate and the elution time for mobile phase or unretained solute when the first baseline disturbance is observed [40]. ^b Peak asymmetry factor, $A_s = \frac{w_R}{w_L}$, where w_R is the distance from the peak midpoint (perpendicular from the peak highest point to baseline) to the trailing edge of the peak and w_L is the distance from the leading edge of the peak to the peak midpoint measured at 10 % of peak height [40]. ^c Plate number, $N_p = 5.54 \left(\frac{t_R}{w_{h/2}} \right)^2$, where t_R is retention time and $w_{h/2}$ is width of the peak at half height [40]. ^d Limit of detection (LOD) was calculated by dividing the standard deviation of peak signals of fortified blank samples close to the expected LODs by the slopes of the corresponding calibration curves [40]. LODs calculated at 254 nm for ascorbic acid and 280 nm for 2,3-DHBA and pyrocatechol.

The calibration curves for both UV and EC detections were plotted using peak heights for each solute (see Fig. S13). The LODs values of EC detection were ~4-25 times lower than those obtained with UV detection). The LODs obtained for the gap-FC were 0.10 μM, 0.09 μM, 0.19 μM for ascorbic acid, 2,3-DHBA and pyrocatechol, respectively (see Table 1).

However, the performances of gap-FC in terms of peak asymmetry, peak efficiency and peak FWHM are compared with UV detector in this platform (see Table 1). The delay time between chromatograms ~1-2s, the loss of efficiency (~9-11%), ~2-3% peak broadening in gap-FC was given by the connecting pathways [41], geometry [42] and the effective volume of FC [40].

3.2.2 Capillary LC-EC

The chromatograms of test solutes using the capillary LC-EC system are shown in Fig. 6.

Fig. 6. Chromatograms showing separations of dopamine, 2,3-DHBA, and pyrocatechol using capillary LC platform. Conditions: mobile phase (v/v=20:80): acetonitrile:25 mM citrate buffer, pH 3.5; elution: isocratic; flow rate: $1 \mu\text{L min}^{-1}$; sample injection loop: i.d: $25 \mu\text{m}$, volume: 174 nL; capillary LC column (injector-capillary inlet): ProteCol C18 RP column (length: 15 cm, i.d: $150 \mu\text{m}$, particles size: $3 \mu\text{m}$), column oven temperature: 35°C ; pressure: ~ 110 bar; capillary (column-gap-FC): i.d $100 \mu\text{m}$, length 15 cm; gap distance: $30 \mu\text{m}$; WE: Pt; and AD applied potential: +800 mV.

Table 2. Comparison of analytical performance of gap-FC with UV detector in capillary LC platform.

Detector	Analyte	Void Volume ^a [μL]	Peak asymmetry factor ^b	Peak efficiency (plate number ^c / column length) [N m ⁻¹]	Peak FWHM [min]	Baseline noise (3SD) 5-10 min	LOD ^d [μM]	RSD [%]
UV	Dopamine	0.2	1-1.2	24800-32200	0.25	0.02 [mAU]	0.20	2.5-
	2,3-DHBA					(254 nm)	0.29	4.5
	Pyrocatechol					0.04 [mAU]	0.19	
Gap-FC	Dopamine	-	1.1-1.3	19200-25100	0.29	0.54 [nA]	1.63	3.2-
	2,3-DHBA						1.96	4.9
	Pyrocatechol						2.66	
a, b, c, d Calculation details are given in Table 1.								

The calibration curves for capillary LC peaks using either UV absorbance or EC detection (not simultaneous) were plotted based on peak height response (see ESI Fig. S10 and Fig. S11). Once again LODs were 1.63 μM, 1.96 μM, and 2.66 μM for dopamine, 2,3-DHBA, and pyrocatechol, respectively, using the gap-FC. In this case, these LODs were at least one order of magnitude higher than those obtained with UV detection (see Table 2). We emphasise the fact that there is literature that reported sub-nanomolar LODs for similar analytes where LODs were expressed as the standard deviations of baseline noise (e.g., Gu *et al.* [43]). In this study, we calculated the LODs as the ratio of the standard deviation of baseline noise to the slope of the corresponding calibration curves [40]. The resulting differences in LODs between the standard and capillary format LC systems reflect the response dependencies characterised within section 3.1. However, the performances of gap-FC in terms of peak asymmetry, peak efficiency and peak FWHM are compared with UV detector in this platform (see Table 2).

Table 3 compares the analytical performance of the gap-FC used here with the standard LC and capillary LC formats, with other reported data for flow-through EC detectors [31, 32]. The LODs for the gap-FC EC detector in standard LC were at least one order of magnitude lower than those obtained in the capillary LC-EC system for 2,3-DHBA, and pyrocatechol. More importantly, the LODs obtained with the gap-FC were ~2 times lower than the EC detectors

reported by Santos *et al.* [31] for ascorbic acid and Pluangklang *et al.* [32] for 2,3-DHBA, and pyrocatechol, respectively (see Table 3).

Table 3. Comparison of performance of gap-FC with other reported EC detectors in flow-based analysis systems.

Platform-EC method	Potential [mV]	Flow rate [mL min ⁻¹]	Solvent (v/v), pH	Linearity [μM]	LOD [μM]	Ref.
FIA-pulsed AD	400	0.6	0.1 M acetic acid/ acetate, pH 4.7	28.5-140	0.11 ^a	[31]
Standard LC-AD	800	0.3	25 mM citrate buffer, pH 3.5:ACN (20:80)	0.25-125	0.18 ^b 0.23 ^c	[32]
Standard LC-AD	800	0.6	25 mM citrate buffer, pH 3.5:water:ACN (20:20:60)	0.50-100	0.10 ^a 0.09 ^b 0.19 ^c	This work
Capillary LC-AD		0.001	25 mM citrate buffer, pH 3.5:ACN (20:80)	10-200	1.96 ^b 2.66 ^c	

^a Ascorbic acid, ^b 2,3-DHBA, and ^c Pyrocatechol.

4. Conclusion

In this study, a new gap-FC has been characterised in terms of detector response, EC conversion efficiency, and effective cell volume for flow-based analysis. The highest reported EC conversion (~11%) at the lowest reported effective cell volume (35 nL) was achieved. The gap-FC provided EC measurements with high repeatability, high sensitivity, and was readily applied within flow-based analytical systems across a wide flow rate range. The analytical performance in terms of sensitivity and LODs values of standard LC-EC was ~4-25 times lower than the capillary LC-UV, capillary LC-EC, and standard LC-UV systems for test solutes, namely ascorbic acid, 2,3-DHBA, and pyrocatechol. Additionally, for the standard LC platform, ~2 times lower LODs were obtained than those reported to-date using alternative EC detectors, such as those based upon TL- and WJ-FCs.

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ACCEPTED MANUSCRIPT

Electronic Supplementary Information (ESI)

Capillary gap flow cell as capillary-end electrochemical detector in flow-based analysis

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The supporting information content includes:

1. Gap-FC: body parts construction, gap distance adjustment, and gap-, TL- and WJ-FC comparisons.
2. FIA platform: instrumentation and calculation of effective cell volume.
3. Separation platforms: capillary LC-UV and LC-EC systems, and standard LC-UV-EC system.
 - 3.1. Capillary LC-UV and LC-EC systems: optimisation of parameters such as mobile phase, buffer pH, flow rate, capillary i.d., gap distance, applied potential and calibration plots.

3.2. Standard LC-UV-EC system: optimisation of flow rate and calibration plots.

1. Gap-FC

Fig. S1. The gap-FC (elevation view)

1.1 Body parts construction

Fig. S2. The gap-FC: (a) connection of capillary, (b) measuring part (1. cone for tightening, 2. screw for coupling screw, 3. hole for tightening of sealing of capillary, 4. hole for capillary insertion), and (c) SPEs connector (1. body part of gap-FC, 2. printed circuit board with contacts,

3. gold plated contacts, 4. supporting desk, 5. insulating spacer, and 6. screw M3 for fastening of printed board with contacts). Dimensions: D7: 0.4 mm, D8: 0.95 mm, D9: 1.52 mm, D10: 5.6 mm, L1: 2.9 mm, L2: 3 mm, L3: 5.9 mm, L4: 7.4 mm, L5: 8.5 mm, L6: 2.54 mm, L7: 2.5 mm, h1: 0.4 mm, and h2: 0.005-0.015 mm.

The visual inspection of bubbles and space around the WE were captured by using an optical microscope (model: AM4113T-GFBW, Dino-Lite digital microscope, USA). Experimental conditions: 1 mM Rhodamine 6G dye (Sigma-Aldrich, USA), flow rate: $10 \mu\text{L min}^{-1}$, LabSmith microsyringe pump, solvent: water, capillary (injector-gap-FC as EC detector): i.d: $100 \mu\text{m}$, and gap distance: $30 \mu\text{m}$. For this purpose, semi-transparent Poly(methyl methacrylate (PMMA) gap-FC was used instead of PEEK material-based gap-FC that used for experimental investigations. See web link: <https://www.youtube.com/watch?v=GIWNc4xCr8E>

1.2 Gap distance adjustment

One of the key features of gap-FC is to facilitate a quick and accurate method to adjust the gap distance between the electrode and capillary orifice. To adjust the gap distance, the commercially available as well as mechanically durable thin steel (part number: 412092, Kincrome Australia Pty Ltd.) with vendor specified thickness was used as a spacer. The spacer was cut into the same diameter of the electrode. In the cell, the spacer was placed between the capillary orifice and electrode. The upper part of the cell and capillary were locked with screws and the built-in system for fastening the capillary. Finally, the upper part of the cell was opened to take out the spacer and locked again that confirms the desired gap distance between the working electrode and the capillary orifice. We used an external marker on the capillary to double check the gap distance by measuring the shift of the marker when the spacer was taken out using an optical microscope (model: AM4113T-GFBW, Dino-Lite digital microscope, USA). We used commercially available steel sheet with thicknesses varying from $30 \mu\text{m}$ (minimum thickness available from vendors) to $100 \mu\text{m}$. We measured the spacer thickness for at least 5 times ($30 \pm 0.02 \mu\text{m}$) before placing the spacer between capillary outlet and electrode inside gap-FC.

2. FIA platform

2.1 Instrumentation

Fig. S3. Schematic flow diagram of μ FIA platform.

2.2 Effective cell volume

Fig. S4. Demonstration of calculating the response times ($t_{h/2}$) in gap-FC and C^4D detectors from the measured peak signals (S_h) at flow rate $5 \mu\text{L min}^{-1}$, capillary length: 7 cm and gap distance: 50 μm . Conditions: solvent: water, flow rate: $5 \mu\text{L min}^{-1}$; solute: 10 nM $[\text{Fe}(\text{CN})_6]^{4-}$ in 0.04 M NaOH; sample injection volume 3 μL ; capillary (injector-gap-FC): i.d 100 μm , length 7 cm; gap distance: 50 μm ; and AD potential: +650 mV. t_0 is initial time, $t_{h/2}$ is time at half of signal height, $S_{h/2}$ is half of signal height, S_h is signal height.

The V_E of gap-FC can be measured by varying the length of the capillary from injector to detector in X-axis and total volume ($V_T = V_I + V_C + V_E$) obtained from the detector signal in Y-axis. V_T is the total of injector volume i.e. void volume of the injector V_I , capillary volume V_C , and V_E . For a given length of the capillary (see Fig. S4), V_T can be calculated from the detector signal by considering time at half of the signal height and flow rate. In X-axis when the length of the capillary is zero, $V_{T-C^4D} = V_I$ and $V_{T-gap-FC} = V_I + V_E$. Finally, V_E of gap-FC can be calculated by subtracting the value of V_I from the value of $V_{T-gap-FC}$ in Y-axis.

Table S1. Total volume calculation of C^4D and gap-FC at capillary length 7 cm and gap distance 50 μm .

Detector	Time at half of peak height, $t_{h/2}$ [s]	Flow rate, Q [$\mu L \min^{-1}$]	Total volume, V_T ($t_{h/2} \times Q$) [nL]
C^4D	7	5	583
Gap-FC	9	5	750

3. Analytical applications

3.1 Capillary LC-UV/LC-EC

3.1.1 Optimisation of mobile phase

Fig. S5. Dependency of solute response on organic solvent ratio. Conditions: mobile phase (v/v=15:85, 20:80, and 25:75): acetonitrile:25 mm citrate buffer, pH 3.5; elution: isocratic; flow rate: 1 $\mu L \min^{-1}$; solute concentration: 80 μM ; sample injection loop: i.d: 25 μm , volume: 174 nL; capillary LC column (injector-UV or EC detector): ProteCol C18 RP column (length: 15 cm, i.d: 150 μm , particles size: 3 μm), column

oven temperature: 35 °C; pressure: ~110 bar; UV absorbance wavelength: 280 nm; capillary (column-gap-FC): i.d 100 μm , length 15 cm; gap distance: 30 μm ; WE: Pt; and AD applied potential: +800 mV.

Fig. S6. Dependency of solute response on pH of mobile phase. Conditions: mobile phase (v/v=20:80): acetonitrile:25 mM citrate buffer, pH 3, and 5; elution: isocratic; flow rate: 1.5 $\mu\text{L min}^{-1}$; solute concentration: 80 μM ; sample injection loop: i.d: 25 μm , volume: 174 nL; capillary LC column (injector-UV or EC detector): ProteCol C18 RP column (length: 15 cm, i.d: 150 μm , particles size: 3 μm), column oven temperature: 35 °C; pressure: ~110 bar; capillary (column-gap-FC): i.d 100 μm , length 15 cm; gap distance: 30 μm ; WE: Pt; and AD applied potential: +800 mV.

3.1.2 Optimisation of flow rate

The different flow rate was investigated by observing peak response in the EC detector. The increase of flow rate increases the pressure of the column, and the pressure of the column was limited by column specification. The pressure ~110, and 150 bar pressures were observed for the flow rate 1 $\mu\text{L min}^{-1}$, and 1.5 $\mu\text{L min}^{-1}$, respectively. The maximum S/N ratio obtained for flow rate 1 $\mu\text{L min}^{-1}$ in EC detector considering pressure limit of the column.

3.1.3 Optimisation of capillary i.d

The maximum S/N ratio obtained for capillary i.d (capillary LC column-gap-FC) 100 μm .

Fig. S7. Dependency of solute response capillary on internal diameter (i.d). Conditions: mobile phase (v/v=20:80): acetonitrile:25 mM citrate buffer, pH 3.5; elution: isocratic; flow rate: $1 \mu\text{L min}^{-1}$; solute concentration: $80 \mu\text{M}$; sample injection loop: i.d: $25 \mu\text{m}$, volume: 174 nL; capillary LC column (injector-UV or EC detector): ProteCol C18 RP column (length: 15 cm, i.d: $150 \mu\text{m}$, particles size: $3 \mu\text{m}$), column oven temperature: 35°C ; pressure: ~ 110 bar; capillary (column-gap-FC): i.d 75, 100, and $150 \mu\text{m}$, length 15 cm; gap distance: $30 \mu\text{m}$; WE: Pt; and AD applied potential: +800 mV.

Table S2. The dependency of solute response on capillary internal diameter (i.d).

Capillary i.d (capillary LC column-gap-FC) [μm]									
Solute	75	100	150	75	100	150	75	100	150
	Peak height [nA]			Peak width [min]			S/N [nA]		
							5 x baseline noise		
							1-2 min [nA]		
Dopamine	111	84	58	0.31	0.36	0.42	11	25	18
2,3-DHBA	113	73	65	0.24	0.32	0.40	12	22	20
Pyrocatechol	127	86	80	0.27	0.36	0.38	13	27	25

3.1.4 Optimisation of gap distance

The maximum S/N ratio obtained for gap distance $30 \mu\text{m}$.

Fig. S8. Dependency of solute response on gap distance. Conditions: mobile phase (v/v=20:80): acetonitrile:25 mM citrate buffer, pH 3.5; elution: isocratic; flow rate: $1 \mu\text{L min}^{-1}$; solute concentration: $80 \mu\text{M}$; sample injection loop: i.d: $25 \mu\text{m}$, volume: 174 nL ; capillary LC column (injector-UV or EC detector): ProteCol C18 RP column (length: 15 cm , i.d: $150 \mu\text{m}$, particles size: $3 \mu\text{m}$), column oven temperature: 35°C ; pressure: $\sim 110 \text{ bar}$; capillary (column-gap-FC): i.d $100 \mu\text{m}$, length 15 cm ; gap distance: $30, 50$, and $70 \mu\text{m}$; WE: Pt; and AD applied potential: $+800 \text{ mV}$.

Table S3. Dependency of solute response on gap distance.

Gap distance (capillary outlet-electrode) [μm]									
Solute	30	50	70	30	50	70	30	50	70
	Peak height [nA]			Peak width [min]			S/N [nA]		
							5 x baseline noise		
							1-2 min [nA]		
Dopamine	114	108	102	0.32	0.36	0.37	42	40	37
2,3-DHBA	61	42	41	0.33	0.40	0.43	20	16	15
Pyrocatechol	59	47	42	0.35	0.38	0.44	19	18	17

3.1.5 Optimisation of applied potential

The maximum S/N ratio obtained for AD applied potential $+800 \text{ mV}$.

Fig. S9. Dependency of solute response on applied potential. Conditions: mobile phase (v/v=20:80): acetonitrile:25 mM citrate buffer, pH 3.5; elution: isocratic; flow rate: $1 \mu\text{L min}^{-1}$; solute concentration: $80 \mu\text{M}$; sample injection loop: i.d: $25 \mu\text{m}$, volume: 174 nL ; capillary LC column (injector-UV or EC detector): ProteCol C18 RP column (length: 15 cm , i.d: $150 \mu\text{m}$, particles size: $3 \mu\text{m}$), column oven temperature: 35°C ; pressure: $\sim 110 \text{ bar}$; capillary (column-gap-FC): i.d $100 \mu\text{m}$, length 15 cm ; gap distance: $30 \mu\text{m}$; WE: Pt; and AD applied potential: $650\text{--}950 \text{ mV}$.

Table S4. Dependency of solute response on applied potential.

Solute	Applied potential [mV]														
	650	725	800	875	950	650	725	800	875	950	650	725	800	875	950
	Peak height [nA]					Peak area [nA min]					S/N [nA]				
Dopamine	72	80	113	105	106	28	32	42	40	35	152	167	238	220	222
2,3-DHBA	59	58	60	59	47	21	24	22	23	17	123	121	126	125	99
Pyrocatechol	64	66	65	66	48	28	30	27	15	16	135	138	136	140	101

3.1.6 Calibration plots

The calibration plots of capillary LC-UV and LC-EC.

Fig. S10. Calibration plots of capillary LC-UV system. Conditions: Mobile phase (v/v=20:80): acetonitrile:25 mM citrate buffer pH 3.5; elution: isocratic; flow rate: $1 \mu\text{L min}^{-1}$; solute concentration: 5-180 μM ; sample injection loop: i.d: 25 μm , volume: 174 nL; capillary LC column (injector-UV detector): ProteCol C18 RP column (length: 15 cm, i.d: 150 μm , particles size: 3 μm), column oven temperature: 35 $^{\circ}\text{C}$; pressure: ~ 110 bar; and UV absorbance wavelength: 280 nm.

Fig. S11. Calibration plots of capillary LC-EC system. Conditions: Mobile phase (v/v=20:80): acetonitrile:25 mM citrate buffer, pH 3.5; elution: isocratic; flow rate: $1 \mu\text{L min}^{-1}$; solute concentration: 10-225 μM ; sample injection loop: i.d: 25 μm , volume: 174 nL; capillary LC column (injector- EC detector): ProteCol C18 RP column (length: 15 cm, i.d: 150 μm , particles size: 3 μm), column oven temperature: 35 $^{\circ}\text{C}$; pressure: ~ 110 bar; capillary (column-gap-FC): i.d 100 μm , length 15 cm; gap distance: 30 μm ; WE: Pt; and AD applied potential: +800 mV.

3.2 Standard LC-UV-EC

3.2.1 Optimisation of flow rate

The maximum S/N values obtained for flow rate 0.6 mL min^{-1} (see ESI Fig. S12).

Fig. S12. Dependency of solute response on flow rate. Conditions: mobile phase (v/v=60:20:20): ACN: water:25 mM citrate buffer, pH 3.5; elution: isocratic; flow rate: 0.2-1.2 mLmin⁻¹; solute concentration: 100 μM; sample injection volume: 10 μL; LC column (injector-UV detector): YMC Pac Pro C18 RP column (length: 25 cm, i.d: 4.6 mm, particles size: 5 μm), column oven temperature: 25 °C; pressure: ~1050 psi (~73 bar); capillary (UV-gap-FC) i.d: 100 μm, length: 7 cm; gap distance: 30 μm; WE: Pt; and AD applied potential: +800 mV.

Table S5. Dependency of solute response on gap distance.

Flow rate [mL min ⁻¹]							
Solute	0.2	0.4	0.5	0.6	0.8	1	1.2
	5 x baseline noise 1-2 min [nA]						
	1.7	2.1	1.9	1.8	2.25	3.7	4.4
	S/N [nA]						
Dopamine	61	77	85	105	92	60	57
2,3-DHBA	40	48	59	67	60	39	37
Pyrocatechol	44	57	66	82	73	47	44

3.2.2 Calibration plots

In standard LC-UV-EC system, the maximum S/N values obtained for flow rate 0.6 mL min⁻¹ (see ESI Fig. S12). The parameters such as capillary i.d (UV-gap-FC) 100 μm, gap distance 30 μm, and AD applied potential +800 mV were

same as used in capillary LC platform. Under these optimised conditions, the calibration curves of UV and EC detector (see ESI Fig. S13) showed the linearity from 0.50-100 μM for all solutes, respectively.

Fig. S13. Calibration plot in standard LC-UV-EC systems. Conditions: mobile phase (v/v=60:20:20): ACN: water:25 mM citrate buffer, pH 3.5; elution: isocratic; flow rate: 0.6 mL min^{-1} ; sample injection volume: $10 \mu\text{L}$; LC column (injector-UV detector): YMC Pac Pro C18 RP column (length: 25 cm, i.d. 4.6 mm, particles size: $5 \mu\text{m}$), column oven temperature: 25°C ; pressure: $\sim 1050 \text{ psi}$ ($\sim 73 \text{ bar}$); UV absorbance wavelength: 254 nm and 280 nm; capillary (UV-gap-FC) i.d: $100 \mu\text{m}$, length: 7 cm; gap distance: $30 \mu\text{m}$; WE: Pt; and AD applied potential: +800 mV.