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Induced sample via transient isotachophoresis mediated with sweeping in micellar electrokinetic chromatography for the dual-stacking strategy of non-steroidal anti-inflammatory drugs in environmental water samples

Nadhiratul-Farihin Semail[a], Aemi Syazwani Abdul Keyon[b], Bahruddin Saad[c], Sazlinda Kamaruzaman[d], Nur Nadhirah Mohamad Zain[a], Vuanghao Lim[a], Mazidatulakmam Miskam[e], Wan Nazwanie Wan Abdullah[e], Muggundha Raoov[f], Noorfatimah Yahaya[a,*]

[a] *Department of Toxicology, Advanced Medical and Dental Institute (AMDI), Universiti Sains Malaysia, Bertam, 13200 Kepala Batas, Penang, Malaysia*
[b] *Department of Chemistry, Faculty of Science and Centre for Sustainable Nanomaterials, Ibnu Sina Institute for Scientific and Industrial Research, Universiti Teknologi Malaysia, 81310 UTM, Johor Bahru, Johor, Malaysia*
[c] *Fundamental & Applied Sciences Department, Universiti Teknologi PETRONAS, 32601 Seri Iskandar, Perak Darul Ridzuan, Malaysia*
[d] *Department of Chemistry, Faculty of Science, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia*
[e] *School of Chemical Sciences, Universiti Sains Malaysia, 11800 USM Pulau Pinang, Malaysia*
[f] *Department of Chemistry, Faculty of Science and Universiti Malaya Centre for Ionic Liquids (UMCIL), Universiti Malaya, Kuala Lumpur, Malaysia*

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ABSTRACT

Realising the need to devise a simple, sensitive, and reliable detection method, this study investigated the development of a dual-stacking transient isotachophoresis (t-ITP) and sweeping in micellar electrokinetic chromatography with diode array detector (t-ITP/sweeping-MEKC-DAD) for the determination of selected non-steroidal anti-inflammatory drugs (NSAIDs); ketoprofen, diclofenac and naproxen from aqueous matrices. Prior to the system setup, various parameters were optimised to assess the potential use of the t-ITP paired with the sweeping stacking technique in micellar background electrolyte for dual preconcentration and separation of trace amounts of NSAIDs. Once the optimum conditions have been established, the method performance was validated and applied to 17 environmental water samples. Based on the results, the combined t-ITP and sweeping approach significantly improved the stacking and separation sensitivity. A large volume of samples could also be introduced and subsequently separated by MEKC with greater focusing effects due to the sweeping. Under optimised conditions, the developed method exhibited excellent linearity at a high range (0.1–500 ng/mL, $r^2 \geq 0.998$), low limits of detection (LODs) of 0.01–0.07 ng/mL, and a remarkable relative recovery (RR) of 99.6–101.9% with a relative standard deviation (RSD) of 1.4–8.6% ($n = 9$). Ultimately, the sensitivity enhancement factors improved up to 666-fold using the optimised method. Therefore, the proposed method presents a simplified yet effective and suitable for the determination of NSAIDs from aqueous matrices.

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1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are universal analgesic medications to treat a wide range of acute and chronic pain disorders in both humans and livestock animals. In the pre-hospital era, patients with moderate to severe pain were prescribed NSAIDs due to their potent anti-inflammatory and antipyretic properties [1] (Fig. S1) in place of prescription drugs that may have side effects. However, the uncontrolled discharge of pharmaceuticals and their metabolites in water bodies has been implicated in the severe impact of bioaccumulation and biomagnification on aquatic life as well as the contamination of drinking water from natural resources. The trace level of NSAIDs residue can easily disperse through water bodies, resulting in adverse health effects, including gastrointestinal bleeding, ulcers, and aplastic anemia [2,3].

* Corresponding author.
E-mail address: noorfatimah.usm@gmail.com (N. Yahaya).

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Recent literature reviews have recognised several NSAIDs as harmful emerging chemical contaminants with the reported concentration in aquatic samples reaching parts per billion (ppb) level (equivalent to ng/mL) [4,5]. In addition, the Australian government established the Guidelines for Water Recycling: Augmentation of Drinking Water Supplies for NSAIDs, which stated the maximum concentration of NSAIDs in drinking water as follows; ibuprofen 28 ng/mL, ketoprofen 0.38 ng/mL, aspirin 2.1 ng/mL, naproxen in 0.57 ng/mL, and diclofenac 0.81 ng/mL [6,7]. Thus, it is crucial to devise a simple, sensitive, and reliable method for the determination of NSAIDs in environmental water samples. At present, the high-performance liquid chromatography (HPLC) [8], gas chromatography (GC) [9], and capillary electrophoresis (CE) [10] are among the various analytical methods that have been developed to determine the level of NSAIDs in environmental water samples. Due to the poor volatility of NSAIDs, both HPLC and CE are more appropriate to detect NSAIDs than GC. Comparatively, the separation of ionisable analytes through CE is more beneficial than HPLC in terms of flexibility, a higher separation efficiency that offers versatile resolving power for complex analysis, ease and simplicity of operation, rapid analysis, cheaper setup, and mostly non-toxic and environmentally friendly reagents [10].

Nevertheless, conventional optical detection methods in CE have a limited sensitivity due to the short optical path length dictated by the internal diameter of the capillaries, and the small injection volume of samples. Although the optical path length can be increased to enhance the CE sensitivity by applying a bubble cell or a Z-shaped cell [11], and by using a coupled column ITP in microchip electrophoresis [12] or using highly sensitive detector, such as mass spectrometer [13], these approaches require either instrument modification or new detector installations, which are uneconomical and require well-trained personnel. In view of this, the development of preconcentration methods through online and/or offline stacking strategies in CE is of great importance to providing a simple, efficient, and sensitive detection. Online preconcentration strategies have been reported to concentrate the target analytes by 10- to 100-folds or even higher via several approaches, such as field amplified stacking (FASS) [14], dynamic pH junctions [15], sweeping [16], and micelle to solvent stacking [17]. The growing interest in the dual-stacking technique in preconcentrating analytes is attributable to its ability to achieve higher sensitivity of enhancement factor of analytes. This technique applies two preconcentration methods concurrently, namely the transient isotachophoresis (t-ITP) and sweeping. The t-ITP method is based on the injection of BGE followed by a leading electrolyte (LE), sample injection and then an appropriate amount of terminating electrolyte (TE), which resulted in a moving zone arrangement while stacking and concentrating the analytes in a confined band in the middle.

In the first stage, the t-ITP induces the focusing effect of the analytes, which were stacked in a confined zone between two moving boundaries with two types of electrolytes. The two types of electrolytes, known as the leading electrolyte solution (having ions with higher electrophoretic mobility (μep) than the targeted analytes) and terminating electrolyte solution (have ions with lower electrophoretic mobility) [18] is shown in Fig. 1A. Thus, a greater sample volume can be introduced with up to 20% of the effective capillary length, resulting in improved signal sensitivity. For the second stage, sodium dodecyl sulphate (SDS) is utilised as micelle in stacking, known as sweeping (Fig. 1B). The analytes are first sandwiched between LE and TE and then focused in front of an LE/TE interface before being swept, forming a more concentrated zone by micelle (Fig. 1C). Generally, the micelles are charged and migrate in the reverse direction of the electroosmotic flow (EOF) towards the anode. Once the positive voltage is applied, the EOF would ultimately transport the micelles toward the cathode given its typically stronger electrophoretic velocity than the micelles inside the capillary. As such, solutes that interact strongly with the micelle would take longer to migrate. With improved analyte resolution, highly hydrophobic solutes that interact more strongly with micelles and are retained longer are finally separated through micellar electrokinetic chromatography (MEKC). The cumulative impact of utilising the dual-stacking preconcentration strategy is the enhanced signal sensitivity factor with a much better resolved analyte peak compared to that using MEKC alone. The proposed stacking strategy could be a potential candidate for monitoring NSAIDs in various matrices and applications.

In this study, a simple and efficient dual-stacking strategy for anion analytes via the t-ITP and sweeping-MEKC with diode array detector (t-ITP/sweeping-MEKC-DAD) method was evaluated to quantify selected NSAIDs in environmental water samples. A number of parameters were optimised and applied to assess the potential use of t-ITP paired with the sweeping stacking technique in micellar background electrolyte (BGE). Under the established optimum conditions, the method was validated using ICH Harmonised Tripartite Guideline [19] and was applied to 17 environmental water samples. The successful development of this method would offer a promising analytical tool with analysis time of less than 15 min, cost-effective, and highly sensitive for practical NSAIDs detection.

2. Experimental

2.1. Chemicals and reagents

Standards of ketoprofen (KET), naproxen (NAP), diclofenac (DIC), and sodium dodecyl sulphate (SDS), as well as 1-octanol, ammonium acetate ($NH_4CH_3CO_2$), sodium chloride (NaCl), sodium hydroxide (NaOH), and HPLC-grade methanol (MeOH) were purchased from Sigma Aldrich (St. Louis, MO, USA). Hydrochloric acid (HCl) was obtained from QreC Asia Chemicals (Selangor, Malaysia). Ultrapure water was generated using a Sartorius Milli-Q system (Göttingen, Germany). Stock solutions of KET, NAP, and DIC (1000 μg/mL) were prepared by dissolving the appropriate amounts of each compound in MeOH. The solutions were kept at 4 °C prior to use. The desired working solution for each standard was prepared daily by diluting the stock solution in MeOH.

2.2. Instrumentation

The separation and detection of NSAIDs were carried out using an Agilent 7100 CE system (Agilent Technologies, Santa Clara, CA, USA) equipped with an ultraviolet diode array detector (DAD). The equipment control and data acquisition of electropherograms were processed using the Agilent Instrument Software. The electrophoresis experiments were performed using an uncoated fused-silica capillary (inner diameter: 50 μm, total and effective lengths: 75 cm and 65 cm, respectively) obtained from Agilent Technologies. Prior to use, the new capillary was conditioned by sequentially flushing with 1.0 M NaOH for 30 min, 0.1 M NaOH for 15 min, water for 15 min, and BGE for 10 min at 50 mbar. Before sample run, the capillary was flushed with water for 10 min and BGE solution for 10 min to maintain the repeatability of the analysis. Between every two sample runs, the capillary was washed with 0.1 M NaOH for 2 min, water for 2 min, and BGE for 3 min at 50 mbar. The BGE was composed of 40 mM ammonium acetate and 10 mM SDS (pH 8; adjusted with 2 M NaOH). All solutions were sonicated and filtered through 0.22 μm syringe-filter membranes (Agilent Technologies) before use.

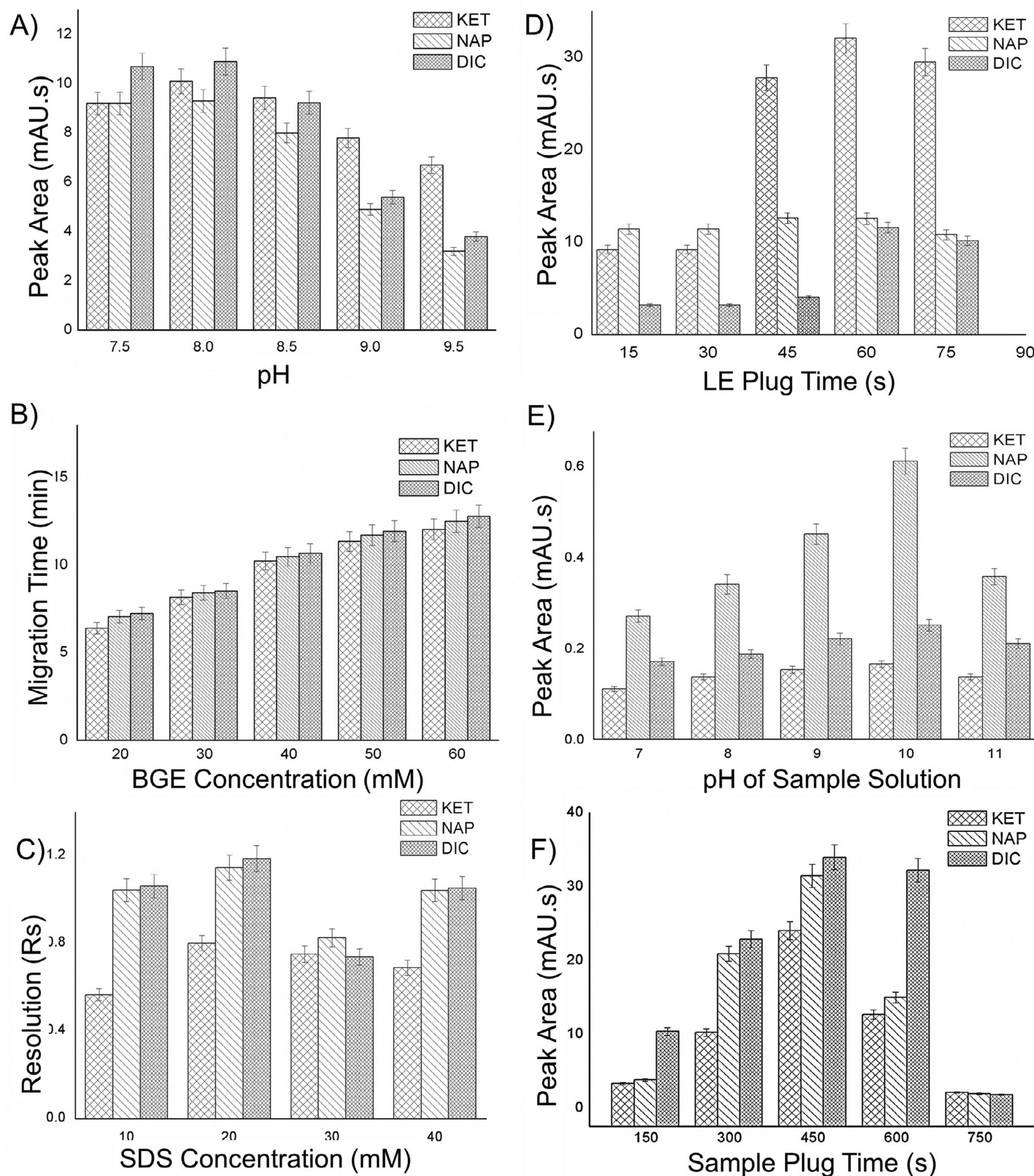

Fig. 1. Dual-stacking strategy by t-ITP- sweeping: (A) transient isotachophoresis, (B)sweeping and (C) micellar electrokinetic chromatography methods for the separation process of NSAIDs.

2.3. Preparation and treatment of samples

A total of 17 environmental water samples (tap water, drinking water, residential water, hospital wastewater, river water, lake water, and seashore water) were collected from different areas in Kedah and Penang, Malaysia. The samples were kept under 4°C until analysis. All water samples were filtered through a 0.22 μm syringe filter and immediately subjected to an optimised dispersive liquid-liquid microextraction (DLLME) procedure [20] with slight modifications. Briefly, 300 µL of 1-octanol was dispersed in 5.0 mL of sample (pH 4) using a Luer lock syringe with needle, followed by sonication for 20 sec and centrifugation for 5 min at 6000 rpm. The supernatant layer (~300 µL) was collected and transferred into a 2 mL snap-lock microtube. Subsequently, the back extraction step [14] of NSAIDs was carried out using 0.3 mL of 4 mM ammonium acetate (pH 10; 10 times BGE dilution without SDS) upon vortex-mixing for 30 sec and centrifugation for 5 min at 6000 rpm before ready for CE analysis.

2.4. Online two-step stacking (t-ITP and sweeping) and sensitivity enhancement factors

In the t-ITP and sweeping method, the BGE solution (40 mM ammonium acetate at pH 8 with 20 mM SDS) was introduced to the capillary at 50 mbar for 120 sec, followed by the hydrodynamic injection (HDI) of 100 mM NaCl as the LE at 50 mbar in 60 sec.

Next, the sample dissolved in 4 mM ammonium acetate (pH 10) was injected hydrodynamically at 50 mbar for 450 s, followed by HDI of 100 mM N-cyclohexyl-2-aminoethanesulfonic acid (CHES) as the TE at 50 mbar for 30 s. The capillary was post-conditioned by flushing with deionised water for 5 min. The stacking and separation of ions were then performed at 20°C and a voltage of +20 kV. Additionally, the sensitivity enhancement factors (SEF) of the developed method were calculated by dividing the detection limits obtained from the t-ITP and sweeping (50 mbar, 450 s) by the detection limits obtained from the conventional hydrodynamic sample injection (50 mbar, 5 sec). All experiments were performed in triplicates. The optimised conditions were selected based on the highest average peak areas obtained for all the studied analytes.

2.5. Method validation

The method of validation applied in this study follows the ICH Harmonised Tripartite Guideline [19] guideline. Primarily, standard calibration curves were established using matrix-matched calibration curve for each NSAIDs was established by serial dilution at a concentration range of 0.1–500 ng/mL. The calibration curves were then constructed by plotting the peak areas versus the concentration of NSAIDs. Each point on the calibration curve represents the mean of nine measurements. The linearity ranges were also evaluated using the coefficient of determination (r^2). In addition, the limits of detection (LODs) and limits of quantitation (LOQs) were calculated based on the signal-to-noise (S/N) ratio of 3 and 10, respectively. Apart from that, the intra-day and inter-day precision were obtained by analysing spiked blank samples at three different concentration levels (1, 25, and 100 ng/mL) and were expressed as the relative standard deviations (RSDs). The intra-day precision was obtained by analysing a sample three times ($n = 3$) in one day, while the inter-day precision was obtained by analysing three samples a day over five consecutive days ($n = 15$). Subsequently, the relative recovery (RR) values were calculated by comparing the obtained amounts of NSAIDs from the samples with corresponding spiked amounts (0.1, 25, and 100 ng/mL ($n = 9$)) on the calibration curve.

3. Results and discussion

3.1. Optimisation of BGE conditions

Several critical variables that affect the BGE were optimised in this study, including type, pH, the concentration of BGE, the concentration of surfactant, and the separation voltage, to achieve satisfactory separation efficiency, selectivity, and peak resolution. During the optimisation process, all other experimental conditions were kept constant. Analyte ions are separated as zones migrating at varying velocities through a BGE or known as a buffering salt solution, in capillary zone electrophoresis (CZE), which is the most extensively used form of CE separation mode. Four types of aqueous BGE, namely ammonium acetate (pKa_1 9.9), sodium tetraborate (pKa_1 *9.14*), sodium acetate (pKa_1 *4.75*), and sodium dihydrogen phosphate (pKa_1 2.15, pKa_2 6.82 and pKa_3 12.38) [21,22], were first evaluated to improve the NSAIDs separation efficiency at a consistent concentration and pH of 20 mM and 7.5, respectively. Based on the separation electropherogram data (Fig. S2), ammonium acetate achieved the highest reproducibility on peak area and migration time (RSD% < 3.4) although a sufficient separation was yet attained. In contrast, the peak sensitivity and resolution of sodium acetate as the separation BGE was somewhat lower than that of ammonium acetate Moreover, both sodium dihydrogen phosphate and sodium tetraborate recorded a low resolution with peak deformation (< 0.5).

The BGE condition was further optimised by identifying the suitable pH value and concentration to enhance the separation quality. The pH of the electrolyte solution is important especially for weak acid or basic analytes since it could affect the charge of analytes which consequently affects the migration rate of the analytes in the electrolyte solution. The NSAIDs investigated in this study were weak organic acids and therefore, the pH of ammonium acetate was adjusted to a range of 7.5–9.5 (Fig. 2A). Practically, higher effective mobility of the compounds implies a faster electrophoretic migration, and, in a proper experimental setup, shortens the analysis time. In other words, a higher pH value reduces the analyte mobility since the high pH level affects the charge of the analytes and the strength of the analyte interaction with the capillary wall [23]. While the separation electropherogram revealed that the peak overlaps at pH 7.5, the peak resolved well (Rs, 1.0) at pH 8 with high reproducibility (RSD% < 3.0). Peak deformation began to appear at a higher alkaline pH range (8–9.5) with lower resolution (0.5) and impaired repeatability on the peak area (RSD% > 20). Additionally, NSAIDs were negatively charged under alkaline conditions due to the deprotonation of the OH group within the NSAIDs structure. This can be explained by the *pKa* values of each NSAID (KET, *pKa* 3.8; NAP, *pKa* 3.9; and DIC, *pKa* 4.0) [24,25]. As a result of a larger EOF in higher alkaline conditions causing a shorter analysis period (< 7 min), they travelled faster (less interaction with the capillary wall) towards the detector end, resulting in overlapping peaks, as opposed to the longer analysis period (15–20 min) under acidic conditions (pH 2.5) [16,26]. In terms of separation quality, the pH of the BGE plays a significant role because the separation of several analytes requires that their effective mobilities differ sufficiently. Hence, the separation quality was improved using ammonium acetate at pH 8 with a short separation time (< 10 min) and was applied to optimise the BGE concentration.

It is well known that the buffer concentration has a direct impact on the Zeta potential of the inner wall of the capillary, which affects the Joule heating, EOF, and current stability during analyte separation [27]. Following the analysis of different compositions of ammonium acetate buffer from 20 to 60 mM, the BGE concentration of 40 mM provided an outstanding separation resolution with the highest peak selectivity and sensitivity (∼ 11 mAU) and appropriate migration time (10 min), as indicated in Fig. 2B. On the contrary, using lower BGE concentrations (< 40 mM) resulted in a high Zeta potential and hence a high EOF, which could easily lead to insufficient separation with poor resolution. The peak heights were also unaffected when the BGE concentration was greater than 40 mM and instead caused higher baseline noise. Furthermore, the analytes retained longer on the inner capillary wall due to their reduced effective mobilities, which corresponded to the long separation time of more than 15 min.

Additionally, higher conductivity solutions cause current instability with the existence of co-migrated peaks (increased peak width), which affects the separation quality and repeatability (RSD% > 8.6). The SEF can be easily achieved by adding a surfactant or micellar solution in BGE as it minimises peak broadening and is applicable for the separation of neutral and charged analytes [28,29]. The use of micellar or surfactant (anionic or cationic) in separation methods by changing the BGE composition has been proven to improve the separation sensitivity (60 times lower LOD) and enhanced the selectivity by manipulating the capability to identify analytes [16,30–32]. The separation is subjected to the individual partitioning equilibrium of the target analytes with micellar and the aqueous phase of BGE [33]. Besides, continuous disaggregation and reconstruction of dynamic micelle formation are able to contain hydrophobic analytes due to the pseudo-stationary phase (PSP) with amphipathic criteria [34].

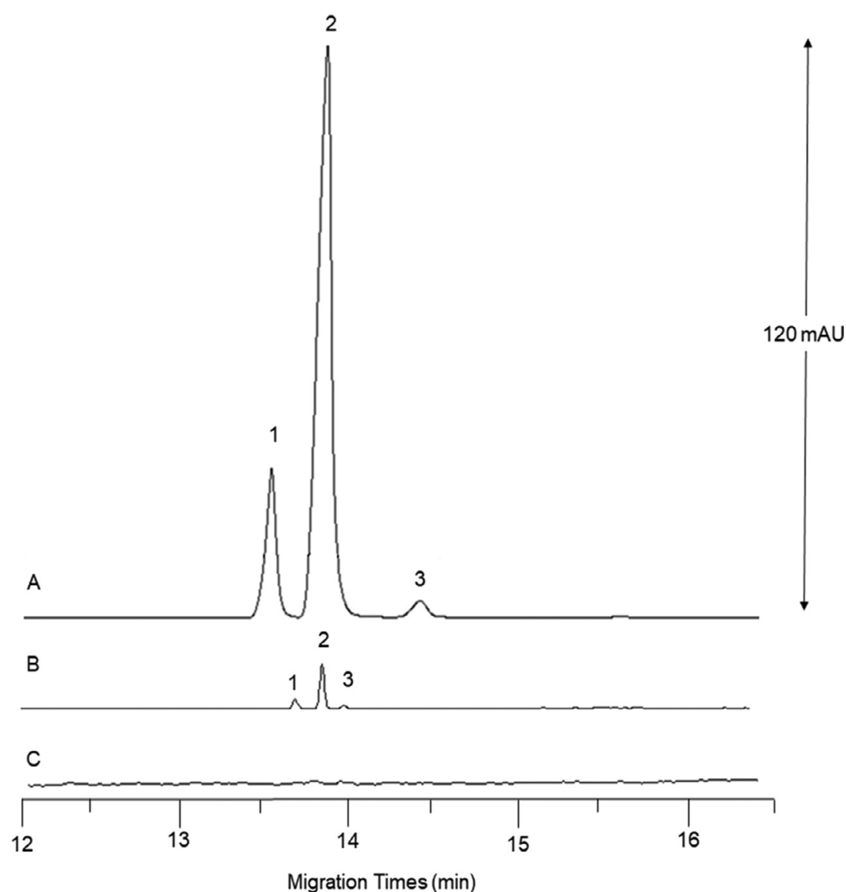

Fig. 2. Effect of several variables on peak area; (A) pH of BGE, (B) BGE concentration, (C) SDS concentration, (D) LE plug time (E) pH of sample solution and (F) injection time ratio.

However, it is difficult to achieve optimal separation selectivity due to the small differences in *pKa* values of all three anionic NSAIDs used in this study, resulting in overlapped peaks under alkaline conditions. Satisfactory resolution of the targeted NSAIDs was obtained when the anionic surfactant SDS was used and added to the BGE. Thus, the BGE of 40 mM ammonium acetate at pH of 8.0 was utilised during the optimisation of SDS concentration from 10 to 40 mM (Fig. 2C). Since the NSAIDs were in anionic form (weak acids), they possessed a weak affinity for negative PSP owing to the electrostatic repulsion that reduced the interaction of the analytes with shorter migration times but the stronger hydrophobic interaction between the analytes and micelles could overcome the repulsion and attraction forces [34,35]. It was found that the increase in SDS concentration caused the resolution to increase since a higher concentration (when $>$ 20 mM SDS was applied) resulted in increased micelle-analyte interactions with longer separation times ($<$ 20 min) [36]. At 10 mM SDS concentration, a fronting peak was observed for three analytes, while higher SDS concentrations ($>$ 20 mM) only instigated excessive current as well as longer analyte migration times with reduced resolution. Considering the achieved selectivity and signal sensitivity ($<$ 60 mAU.s), the SDS concentration of 20 mM was selected and applied in the subsequent experiments. Therefore, the adopted BGE conditions for optimal separation of NSAIDs were 40 mM ammonium acetate buffer at pH 8.0 with 20 mM SDS and +20 kV as the separation voltage.

3.2. Optimisation of t-ITP and sweeping method

In order to achieve higher sensitivity, resolution, and optimal conditions of the t-ITP/sweeping-MEKC-DAD approach, several variables of the t-ITP were investigated, including, LE and TE injection time, pH of sample solution, and sample plug length. The stacking technique (t-ITP) was initiated by infusing the pre-treated capillary with 100 mM NaCl as the LE (60 sec at 50 mbar). Next, a long plug of analytes, which were dissolved in 4 mM BGE without SDS (4 mM ammonium acetate at pH 10), were hydrodynamically injected into the capillary for 300 sec. After the sample plug, 100 mM CHES was introduced as the TE (30 sec at 50 mbar). Once the separation voltage was applied, the diffuse band of NSAIDs introduced during the HDI was fixed by t-ITP between the leading ion (Cl^-) and the terminating ion ($C_8H_{16}NHSO_3{}^-$), after which it left the sample zone and swept, and the separation was performed by MEKC.

The LE and TE for this study were selected based on their charge and electrophoretic mobility. The chloride ion (Cl^-) has higher electrophoretic mobility (64×10^{-9} m^2 v^{-1} s^{-1}) compared to that of CHES (25×10^{-9} m^2 v^{-1} s^{-1}) and a sequential approach was optimised based on the effect of LE and TE plug time on the separation quality of NSAIDs [37–39]. The optimal plug length of both LE and TE ions is crucial since the plug time affects the stacking process quality, which directs the migration range of ions in the capillary. LE plug with higher mobility was first optimised by introducing 100 mM NaCl into the capillary hydrodynamically at a range of 15 to 75 sec at 50 mbar (Fig. 2D). Secondly, TE with a plug length of 100 mM CHES was varied in sequence within the same range as LE. The data showed that the drugs were not fully stacked with LE and TE plugs under 30 sec. A lower value of the peak area was observed at 15 sec for both LE plugs, while the peak area slightly differed and began to increase with higher plug volume. However, the stacking efficiency started to decline after 60 sec with broader and shorter signals

Table 1
Method validation parameters were obtained for spiked river water samples under the optimised t-ITP/sweeping-MEKC-DAD.

NSAIDs	Linearity range (ng/mL)	Regression equation	Coefficient of determination, r^{2a}	Limit of detection (ng/mL)	Limit of quantification (ng/mL)	Sensitivity enhancement factor (SEF) LOD with t-ITP/sweeping-MEKC-DAD /LOD with conventional injection (5 s, 50 mbar)
Ketoprofen	0.1–500	y= 3.974x + 1.205	0.999	0.05	0.16	398
Naproxen	0.1–500	y= 17.163x + 0.469	0.998	0.01	0.03	620
Diclofenac	0.1–500	y= 1.487x + 2.698	0.998	0.07	0.24	666

with irreproducible results. The higher LE/TE plug length resulted in inadequate capillary separation length after the analyte was destacked and left the sample zone [18]. Thus, the optimum LE/TE plug length was 60 sec and 30 sec at 50 mbar, respectively, with the highest signal separation sensitivity with an analyte mixture of 0.1 µg/mL.

The effect of the pH of the sample solution on both the peak capacity and efficiency of stacking was observed in the range between pH 7 (unmodified) and 11. As shown in Fig. 2E, the stacking capacity and efficiency significantly improved with higher peak height when the pH value reached 9–10. As soon as the pH of the sample solution changed to 11, a decrease in the peak height of the stacking process was observed. The sensitivity of the stacking technique was substantially influenced by the acidity of the sample solution while being swept since the target analytes were weak acids (*pKa*; 4–6) and highly dependent on the dissociation rate of the sample solution [40,41]. Accordingly, pH 10 was selected as the optimum pH sample solution and applied as the Back Extract Solution (BES) for the sample extraction.

HDI was employed for the sample introduction since the increase in sample volume enhanced the signal strength. The optimal volume of the injected sample was evaluated from 150, 300, 450, 600, to 750 s, respectively, at a constant pressure of 50 mbar (Fig. 2F). NSAIDs were dissolved in 4 mM BGE without SDS (4 mM ammonium acetate at pH 10) and hydrodynamically injected into the capillary with a simultaneous gradual increase of the plug length. Based on the results, the peak area of the analytes rose as the injection period increased from 150 to 450 s, indicating that a higher injection volume increased the signal sensitivity (< 35 mAU.s). When the injection period was extended from 600 and 750 s, KET and NAP began to exhibit poor resolution with a decreased peak area (< 15 mAU.s) and height owing to the overloading and disruptive focusing effect with an enlarged concentrated zone. Beyond 750 sec, the signal rapidly diminished for all three NSAIDs due to the poor stacking effect caused by the rise in Joule heating. The finalised conditions for the t-ITP/sweeping-MEKC-DAD approach for the separation of NSAIDs were as follow: a BGE of 40 mM ammonium acetate buffer with 20 mM SDS, pH 8.0 (50 mbar for 120 s), LE solution of 100 mM NaCl (60 sec at 50 mbar), a sample matrix of 4 mM ammonium acetate at pH 10.0 (50 mbar for 450 s), TE solution of 100 mM CHES (30 sec at 50 mbar), and a separation voltage of +20 kV.

3.3. Focusing mechanism of the dual-stacking t-ITP/sweeping-MEKC-DAD of NSAIDs

In this study, the extent of the preconcentration factor was analysed based on the sample injected by pressure and stacked via the t-ITP approach, which was mediated by sweeping with micellar BGE synchronously. As displayed in Fig. 1A, the focusing mechanism commenced with the introduction of 40 mM ammonium acetate with 20 mM SDS as the micellar BGE, followed by BGE co-ion (Cl^-) (100 mM NaCl) as the LE, sample solution in diluted BGE without SDS via HDI (4 mM ammonium acetate), and low mobility BGE co-ion ($C_8H_{16}NHSO_3{}^-$) (100 mM CHES) as the TE that established the t-ITP.

The t-ITP approach was capable of sustaining stable stacked boundaries of the sample solution in between with an adequate amount of both leading and terminating co-ion with sample solution mobility in between LE and TE [42]. The chromatographic partitioning, binding, or sorption interaction between the micelles and hydrophobic analytes that enhanced the separation of mixed analytes increased the micelle focusing. The solute would take longer to migrate as the interaction between the solute and micelles increased. Moreover, solutes with higher hydrophobicity interact more strongly with the micelles and are retained longer with improved peak resolution [43]. The stacking effect from the t-ITP boundary itself was possibly contributed by the competency in longer sample plug introduction up to 20% of the effective capillary length, resulting in higher sample volume and improved signal sensitivity.

Additionally, the analyte focusing tendency provided through the active electro-dispersion moving in a bracketed zone which slowed down as it reached the BGE zone due to the different conductivities [44]. The anionic micelle moves from the anode skimming the analyte behind the sample zone concurrently once the voltage is applied due to the strong EOF pushed analyte to the detector end (Fig. 1B) [44–46]. As a result, both dominating factors, the t-ITP boundaries, and the micelle focusing effect simultaneously improved the SEF of the analytes, which was further separated by the MEKC mode (Fig. 1C) with EOF as the major driving force that pushed the analyte-focused-micelle to the detector end. In short, the dual-staking successfully improved the SEF with a highly resolved peak and enhanced signal sensitivity compared to that of the normal injection mode (HDI) in MEKC, as shown in Fig. 3A.

3.4. Method performance analysis

The dual-stacking t-ITP/sweeping-MEKC-DAD method was validated in terms of the linear dynamic range, r^2 value, LOD, LOQ, precision, and recovery. The SEFs based on the LOD of both normal injection HDI-MEKC and t-ITP/sweeping-MEKC-DAD were also determined. Table 1 indicates the method validation parameters for spiked river water samples under the optimised t-ITP/sweeping-MEKC-DAD conditions. The linear dynamic range of a matrix matched calibration from 0.1 to 500 ng/mL was plotted for the spiked sample concentrations and was performed in quintuples ($n = 9$). The LOD and LOQ based on the S/N ratio of 3 to 10 for all targeted NSAIDs were 0.01–0.07 ng/mL and 0.03–0.24 ng/mL, respectively. Furthermore, the intra- ($n = 3$) and inter-day ($n = 15$) precision of peak area and migration time were assessed based on the RSDs values obtained on the same day and five consecutive days, respectively (Table 2). The obtained satisfactory RSDs for intra-day and inter-day were 2.61–8.61, 6.36–11.46 for the peak area and 2.33–9.48, 6.93–10.79 for migration time, respectively. In addition, the SEFs obtained for NSAIDs using the t-ITP/sweeping-MEKC-DAD method were up to 666-fold compared to the LOD in both normal HDI-MEKC modes (Fig. 3). Apart from that, the RR

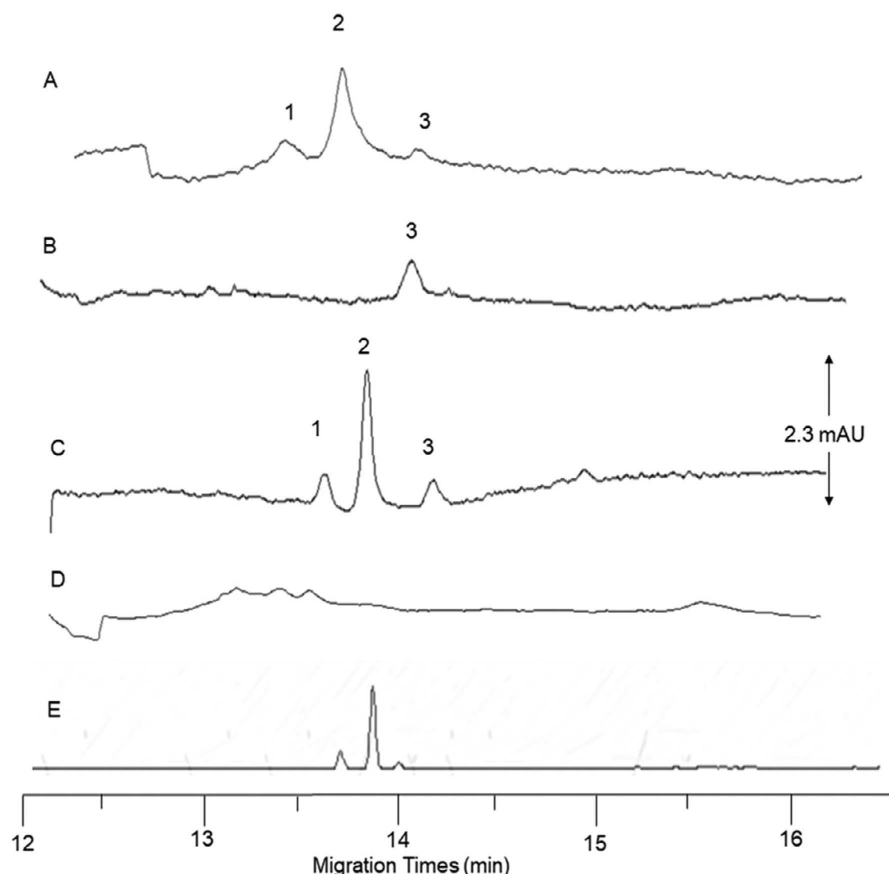

Fig. 3. Electropherograms of spiked sample at 100 ng/mL (A) DLLME- t-ITP- sweeping, (B) DLLME-HDI and (C) blank samples matrices in MEKC-DAD. CE conditions: 40 mM ammonium acetate with 20 mM SDS, pH 8.0, LE solution. 100 mM NaCl (60 s, 50 mbar), sample matrix 4mM ammonium acetate, pH 10.0 (450 s, 50 mbar), TE solution of 100 mM CHES (30 s, 50 mbar) and a separation voltage of +20 kV. Peak identification; (1) ketoprofen, (2) naproxen and (3) diclofenac.

Table 2
Intra-day, inter-day and relative recovery study for NSAIDs at three different spiked levels in water samples.

NSAIDs	Concentration (ng/mL)	Relative recovery (RR) (%) ± RSD (%) (n = 9)	Intra-day (RSD%) (n=3)		Inter-day (RSD%) (n = 15)	
			Peak area	Migration time	Peak area	Migration time
Ketoprofen	1	96.6 ± 3.5	8.43	4.03	11.46	8.09
	25	100.7 ± 7.9	7.95	7.07	10.38	9.42
	100	100.8 ± 2.4	4.85	6.93	7.66	6.93
Naproxen	1	99.9 ± 3.9	4.54	3.79	7.84	8.89
	25	101.9 ± 8.3	2.61	6.91	6.36	9.92
	100	100.0 ± 7.6	7.63	8.45	7.98	7.58
Diclofenac	1	101.2 ± 1.4	7.91	2.33	10.34	8.98
	25	100.5 ± 6.4	6.09	7.33	7.57	10.79
	100	100.1 ± 8.6	8.61	9.48	10.17	7.89

Table 3
Quantitation data of selected NSAIDs for collected water samples.

Sample	No. of analysed sample	No. of positive samples	NSAIDs concentration (ng/mL)		
			Ketoprofen	*Naproxen*	*Diclofenac*
Tap water	1	0	n.d.	n.d.	n.d.
Drinking water	1	0	n.d.	n.d.	n.d.
Residential water	6	2	n.d.–0.3	n.d.–0.1	n.d.–1.8
Hospital wastewater	2	2	n.d.–1.5	n.d.–1.0	n.d.–1.1
River/ Lake/ Seashore water	7	4	n.d.– 0.4	n.d.–0.29	n.d.–1.3

of surface water samples were calculated and determined at three different concentrations levels of 1, 25, and 100 µg/mL (Table 2). Each concentration level and the blank sample were prepared in triplicates and injected three times (n = 9). Based on the results, the RR range of 96.6–101.9% was obtained for all analytes from the environmental water samples.

3.5. Analysis of environmental water samples

The applicability of the proposed method was done by analysing 17 environmental water samples, including tap water (1), drinking water (1), residential area wastewater (6), hospital wastewater (2), and river/lake/seashore water (7). Based on

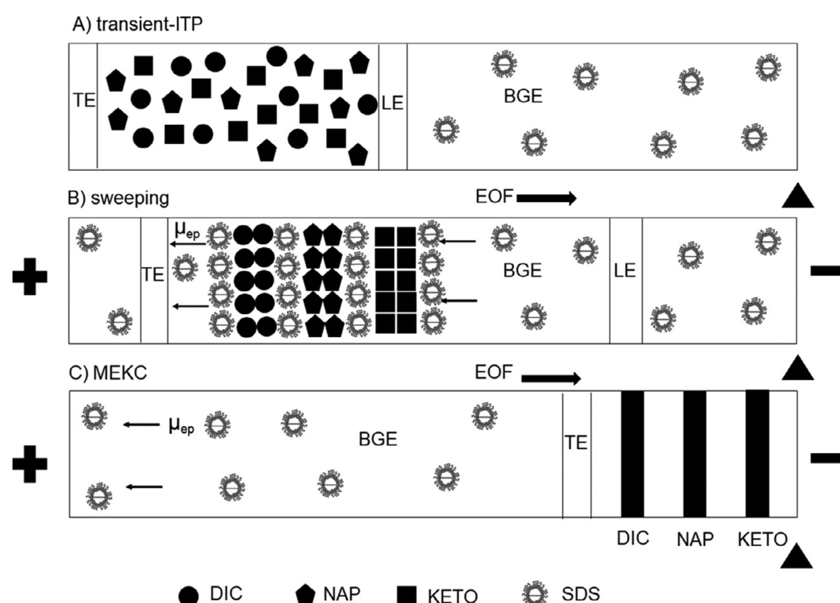

Fig. 4. Electropherograms of raw samples (A) environmental water, (B) residential wastewater, (C) hospital wastewater, (D) tap water and (E) spiked blank sample in 0.5 ng/mL. Peak identification; (1) ketoprofen, (2) naproxen and (3) diclofenac.

Table 4
Comparison of the proposed t-ITP/sweeping-MEKC-DAD with other published methods for the determination of NSAIDs in water samples.

NSAIDs	Preconcentration strategy	Method	Linear range (ng/mL)	Recovery (%)	LOD (ng/mL /µg/kg)	SEF	Refs.
Ibuprofen, Fenoprofen, Naproxen, Ketoprofen, and Diclofenac sodium.	SPE	Field amplified sample injection with reverse migrating micelles capillary electrophoresis diode array detector	0.2–20	70–100	0.1–1.6	63–263	[30]
Ibuprofen, Fenoprofen, Naproxen, Diclofenac Sodium, Ketoprofen, Diflunisal and Indomethacine	Filtration-0.45µm nylon membrane	CF-EKS-CE-DAD	n.a.	52.2–84.8	10.7–47.0	11800	[39]
Diclofenac, Ketoprofen, indomethacin, Ibuprofen, Naproxen and flurbiprofen	Acidification-SPE	Field amplified sample injection with microemulsion electrokinetic chromatography	5–500	92.0–97.3	0.03–0.3	1400–6100	[48]
Diflunisal, Naproxen, Ketoprofen, Indoprofen and Indomethacin	Acid treatment and LLE	Sweeping micelle to solvent stacking capillary electrophoresis UV	600–26 000	94.0–100.0	1230–11720	17–33	[43]
Ibuprofen, Fenoprofen, Naproxen, Diclofenac Sodium, Ketoprofen	Acidification-SPE	Sweeping micellar electrokinetic chromatography diode array detector	10–500	n.a.	1.4–2.5	143–301	[16]
Piroxicam, Ketoprofen, Meloxicam, Naproxen, Diclofenac, Indometacin, Mefenamic Acid and Tolfenamic Acid	µSPE	Ultra-high-performance liquid chromatography tandem mass spectrometry	0.02–50.0	76.5–95.9	0.01–0.19	n.a.	[3]
Naproxen, Diclofenac and Ibuprofen	Molecularly Imprinted Polymers-SPE	High-performance liquid chromatography mass spectrometry	5–3000	99	0.2–1	n.a.	[49]
Ketoprofen, Naproxen and Diclofenac	DLLME	t-ITP/sweeping-MEKC-DAD	0.1–500	96.6-101.9	0.01–0.07	398–666	This work

the results, the concentration of NSAIDs in the water samples (residential, hospital wastewater and environmental water samples) were higher than the maximum residue limits (MRLs) established by the Australian Guidelines in water sample; KET, 0.38 ng/mL, NAP, 0.57 ng/mL, and DIC, 0.81 ng/mL [6]. The quantitation data of NSAID occurrence in five different water matrices is shown in Table 3. The highest average concentration of the selected NSAIDs was recorded from hospital wastewater (∼1.0 ng/mL), which was probably related to the high discharge of human medication and excretion of native compounds [47] compared to residential and environmental water samples. These pharmacological matrices can infiltrate deeper into groundwater sources, where some of the water flow toward nearby streams and lakes. Pharmaceutical traces in wastewater might also infiltrate the circulation systems and would directly contaminate the source of drinking water. Fig. 4 showed the electropherograms of positive samples and spiked blank samples with 0.5 ng/mL NSAIDs and analysed by t-ITP/sweeping-MEKC-DAD.

3.6. Comparative analysis with previously reported methods

The analytical characteristics of the proposed online stacking method were compared with other published methods for the determination of NSAIDs in environmental water matrices, as tabu-

lated in Table 4. The LODs obtained in this study were lower compared to other published works using field amplified sample injection with reverse migrating micelles (FASI-RMM) [30], counter flow electrokinetic supercharging (CF-EKS) [39], field amplified sample injection with microemulsion electrokinetic chromatography (FASI-MEEKC) [48], sweeping with micelle to solvent stacking (sweeping-MSS) [43], and sweeping-MEKC [16] with an optical detector in water samples. In addition, the detection capability or sensitivity of the proposed method in this study was comparable to the reported solid-phase extraction (SPE) followed by high performance liquid chromatography with tandem mass spectrometry (SPE-HPLC-MS/MS) [3,49], which required a higher operational cost than optical detectors. Although SPE procedures offer high detection selectivity and sensitivity, the offline preconcentration technique is relatively costly since the SPE cartridges non-recyclable. Moreover, a higher amount of organic solvent is also required for the conditioning, washing, and elution processes in the SPE methodology. In contrast, the minimum amount of sample and solvent used in this study have highlighted the simplicity and cost-effective features of this technique. Overall, the preconcentration method using the proposed t-ITP/sweeping-MEKC-DAD method significantly improved the sensitivity of analytes and enabled the detection in range of 0.01-0.07 ng/mL.

4. Conclusion

This study established the development of the dual-stacking t-ITP/sweeping-MEKC-DAD method for the determination of three selected NSAIDs in environmental water samples. The offline DLLME and online stacking methods enabled the analytes to be detected in the range of 0.01 to 0.07 ng/mL due to their remarkable preconcentration and enrichment capabilities. Additionally, the method demonstrated exceptional sensitivity, excellent linearity, acceptable precision, and satisfactory accuracy. The advantages of this method were proven by the significant improvement in sensitivity (SEF < 666-folds) and the highly automated system. The sensitivity could be further enhanced by combining this method with other offline or online preconcentration techniques to increase the detectability of trace NSAIDs by CE-DAD in highly complex matrices. Therefore, the t-ITP/sweeping-MEKC-DAD method is a promising alternative for monitoring NSAIDs in various matrices and applications.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest or personal relationships that could have seemed to influence the reported work in this paper.

CRediT authorship contribution statement

Nadhiratul-Farihin Semail: Methodology, Formal analysis, Investigation, Writing – original draft. **Aemi Syazwani Abdul Keyon:** Writing – review & editing, Supervision. **Bahruddin Saad:** Writing – review & editing, Supervision. **Sazlinda Kamaruzaman:** Writing – review & editing. **Nur Nadhirah Mohamad Zain:** Writing – review & editing. **Vuanghao Lim:** Writing – review & editing. **Mazidatulakmam Miskam:** Writing – review & editing. **Wan Nazwanie Wan Abdullah:** Writing – review & editing. **Muggundha Raoov:** Writing – review & editing. **Noorfatimah Yahaya:** Methodology, Writing – original draft, Writing – review & editing, Supervision, Funding acquisition.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2022.463616.

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