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Large-volume sample stacking of selected drugs of forensic significance by capillary electrophoresis

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Abstract

Large-volume sample stacking capillary electrophoresis (LVSS–CE) and conventional capillary electrophoresis (CE) are compared for the separation of drugs of significance to forensic and clinical analyses. LVSS–CE for cations requires the use of an electroosmotic flow (EOF) modifier in conjunction with polarity switching to effect on-column concentration of an analyte and its subsequent migration in the capillary. The run buffer consists of 0.05 mol dm^{-3} disodium tetraborate adjusted to pH 2.2 with orthophosphoric acid, and the EOF modifier is 0.002 mol dm^{-3} cetyltrimethylammonium bromide. CE investigations used an identical run buffer minus the EOF modifier. LVSS–CE and CE investigations used injection times of 30 s and 3 s, respectively. Both modes of capillary electrophoresis are compared in terms of their limits of detection, efficiency, resolution and reproducibility. LVSS–CE is also applied to the analysis of a spiked urine sample.

Keywords: Forensic analysis; Narcotine; Clenbuterol; Flurazepam; Codeine; Pethidine; Hydroxyethylflurazepam; Desalkylflurazepam

1. Introduction

Capillary electrophoresis, a powerful method for the separation of small charged molecules, has been pioneered by several groups [1–4] in the last three decades. It uses narrow-bore capillaries to perform very high efficiency separations. Almost all commercial instruments utilize UV–Vis detection, the LOD of which is hindered by the short optical path necessitated by the column diameter. Typical column dimensions are 75 cm in length by 75 μm internal diameter, leading to an internal volume of just a few microlitres. The maximum allowed injection volume, V_{inject}, to avoid noticeable peak broadening is given by Eq. 1:

$$V_{inject} = Q \cdot V_{column} / N^{1/2} \quad (1)$$

where V_{column} is the column volume, Q is the fraction of allowable peak broadening and N is the number of theoretical plates for the separation. If N is one million, and allowing 5% zone broadening, the maximum injection volume is 225 pl occupying a 100-μm plug in a 50-μm column. This combination of minute injection volume and short pathlength UV–Vis detection put severe restrictions on detection sensitivity. Although the mass limit of detection in capillary zone electrophoresis (CZE) can be very low, because of the small volumes, the concentration limit of detection is usually on the order of 10^{-5} to 10^{-6} mol dm^{-3}, much higher than for corresponding analyses with HPLC. Increasing the injection volume does indeed lead to a limited decrease in LOD, but

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this is countered by increased peak width and impaired separation. Previous groups [5] have acknowledged that, as it stands, the application of CE to situations of trace level forensic or clinical analysis is limited by its relatively high detection limit, requiring pre-injection concentration. For example, in the case of clenbuterol doping of cattle, clenbuterol levels in hair are of the order of 5–15 ng/g of hair [6], concentrations that are not detectable by conventional CE. If CE is to be of use in this type of case, ways have to be found of concentrating the sample on the column. CZE–isotachophoresis has been reported for analyte concentration [7,8]. In this case the sample zone is sandwiched between a leading and a terminating buffer. By applying a voltage, the analytes and the other sample constituents are separated according to their electrophoretic mobilities and focused according to the molarity of the leading buffer.

Sample stacking is an on-column concentration technique for CE. A plug of low concentration buffer, or more commonly water, containing the sample for separation, is injected onto the column filled with run buffer. A high voltage is then employed to effect separation. The low ionic strength sample and the high ionic strength run buffer produce a voltage divider causing an isotachophoretic effect. Most of the voltage is dropped across the sample region, and therefore the electric field is highest in this region. This high electric field drives the ionic components of the sample plug toward the higher ionic strength run buffer region. As the ions experience a lower electric field in the run buffer than in the sample plug, the velocity of the ions decreases as they cross the sample plug boundary causing them to stack. The stacking mechanism occurs for both positively and negatively charged species with the positive species stacking up in front of the sample plug and the negative species at the back. This continues until the components of the sample plug are concentrated, so that the conductivity and electric field within the plug equal that of the run buffer. The analytes can then undergo electrophoretic migration and separation as normal. Stacking can decrease the limit of detection by a factor of three, but is obviously limited by the size of the sample plug that can be injected.

To increase the amount of sample injected onto the column, whilst not impairing separation or increasing peak width, a very large volume of sample can be injected onto the column with the sample solvent being removed after stacking. This can be carried out for charged species by utilizing the EOF. When two phases are placed in contact, a difference in potential develops between them. If one of the phases is a solid, e.g. silica, and the other is a polar liquid, e.g. water, the molecules of the latter will tend to be oriented in a particular direction at the interface and this will generate a double layer and an accompanying potential difference. Cations in the run buffer in CE will have a tendency to associate themselves with the oxygen atoms of the silanol groups. The potential difference between the two phases is known as the ζ (zeta) potential. On application of a potential difference to the capillary in CE, the cations of the run buffer move in the direction of the cathode, giving rise to the driving force behind separations in capillary electrophoresis i.e. the EOF.

Surfactants such as cetyltrimethylammonium bromide (CTAB), when added to the run buffer at very low concentrations, can reverse the EOF [9]. They are used as adsorbates at the solid–liquid interface to control the surface charge [10,11]. Fig. 1a shows that, in the absence of CTAB, the silica wall exhibits an overall slightly negative charge resulting in EOF in the cathodic direction. At low concentration (Fig. 1b), the surfactant behaves like a positive ion and adsorbs on the solid surface resulting in no EOF. At a certain critical concentration of surfactant (Fig. 1c), there is a sudden change in ζ potential, due to association of the CTAB long chain hydrocarbon tails, and a rapid increase in the adsorption density which is attributed to hemimicelle formation. These hemimicelles are two-dimensional micelles formed on the oxide surface at concentrations far below the critical micellar concentration (c.m.c.). The reversal of the ζ potential thus changes the direction of EOF.

2. Experimental

2.1. Reagents and analytes

All solvents were of HPLC-grade or better. Cetyl-

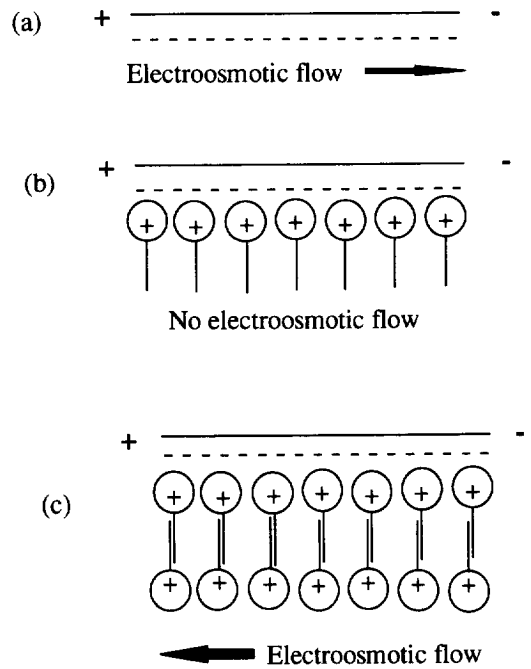

Fig. 1. Effect of CTAB on CZE separations. (a) No CTAB, (b) <0.001 mol dm^{-3} CTAB and (c) >0.001 mol dm^{-3} CTAB.

trimethylammonium bromide, disodium tetraborate and orthophosphoric acid were purchased from Aldrich Chemicals (Gillingham, UK). Methanol was obtained from Rathburn Chemicals (Walkerburn, UK). Benzodiazepine drug samples were obtained from Roche Products (Welwyn Garden City, UK) and other drug samples were obtained from the Northern Ireland Forensic Science Laboratory (Carrickfergus, Northern Ireland, UK). Drug structures are shown in Fig. 2.

2.2. Preparation of analytical solutions

A 10^{-3} g cm^{-3} stock solution of each drug was prepared in methanol, and dilutions (down to 1×10^{-7} g cm^{-3}) were obtained from this solution for limit of detection studies.

2.3. Apparatus and procedures

All experiments were performed on a SpectraPhoresis 1000 (ThermoSeparation Products, Stone, Staffordshire, UK) equipped with a UV–Vis

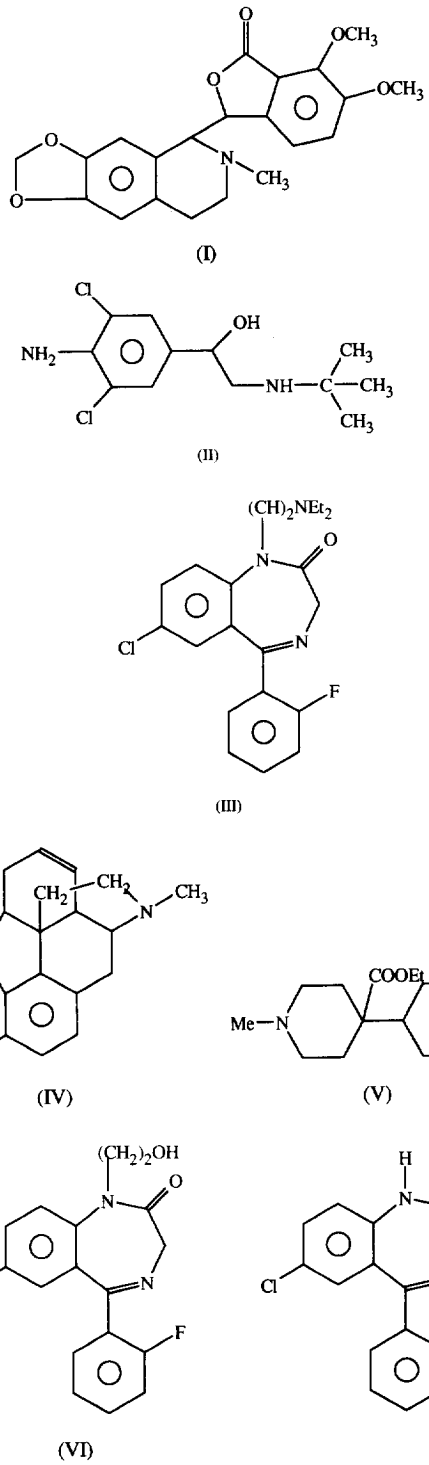

Fig. 2. Structure of drug molecules.

fast scanning detector. All equipment control and data handling were performed using SpectraPhoresis software. Separations were carried out in a 70 cm× 75 μm uncoated fused-silica capillary (Composite Metal Services, Hallow, UK), for which the length of the capillary, from the injector to the detector, was 63 cm. Before CE separations, new capillary columns underwent a conditioning/equilibration procedure consisting of purging with 0.1 mol dm^{-3} NaOH at 60°C for 5 min (to deprotonate the silanol groups) and with water at 60°C for 5 min. This was followed by equilibration with run buffer for 5 min and with a series of "no-injection" injections, whereby a voltage was applied for 5 min. The capillary column was washed for 5 min with buffer before each injection. To prevent capillary blockage, buffers and sample solutions were filtered using 13 mm diameter discs with a pore size of 0.45 μm (Gelman Sciences, Ann Arbor, MI, USA). Samples were injected using the hydrodynamic mode via a vacuum-controlled system with an injection time of 30 s, for large-volume sample stacking studies, and 3 s, in conventional CE. In large-volume sample stacking CE, the sample solvent was removed by applying a positive voltage of 25 kV. The electric current was initially low and the resistance was high, due to the presence of sample solvent. The current was allowed to increase until it had reached 95% of the pre-injection level, and the polarity of the electrodes was reversed. Separation was carried out at −15 kV and 20°C. In conventional CE, separations used a voltage of +25 kV and a temperature of 20°C.

The buffer for CE studies consisted of 0.05 mol dm^{-3} disodium tetraborate, adjusted to pH 2.2 with orthophosphoric acid. Large-volume sample stacking studies additionally used 0.002 mol dm^{-3} CTAB for the purpose of reversing the EOF.

3. Results and discussion

3.1. LVSS–CE

One of the necessary limitations of this mode of capillary electrophoresis is that, if an organic analyte is to be stacked, it must exist in the protonated state at the pH of the run buffer. During the actual stacking process, the analyte must migrate in the opposite direction to the EOF. Neutrally charged species will migrate in the same direction and with the same velocity as the EOF and hence do not stack. Organic analytes with a relatively high pK_a can exist in a state of protonation for a low pH run buffer, so that, when stacking occurs, they will move to the front of the water plug compared to neutral molecules. However, when the EOF is reversed by reversing the electrode polarity, the positively charged species have an electrophoretic affinity for the cathode and attempt to move counter to the direction of the EOF. When the pK_a values are in the vicinity of the pH of the run buffer, the analyte with the lowest pK_a value emerges from the column first – the exact reverse of the elution order for conventional CE. LVSS–CE separation of the five drugs is shown in Fig. 3a. The remnants of the water plug behave as a neutral marker, emerging from the

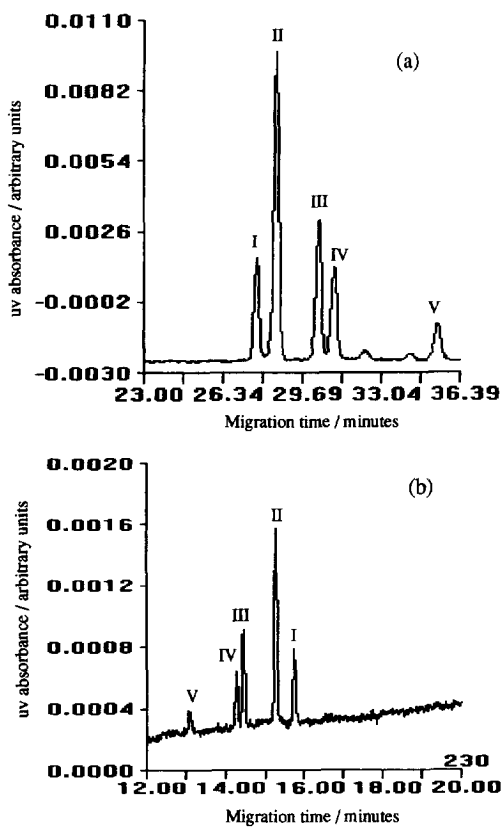

Fig. 3. (a) LVSS–CE electropherogram of narcotine (I), clenbuterol (II), flurazepam (III), codeine (IV) and pethidine (V); (b) CE electropherogram of the same mixture.

column after ca 14.5 min. Narcotine (I) emerges from the column first, followed by clenbuterol (II), flurazepam (III), codeine (IV) and pethidine (V). Table 1 shows the pK_a and the charge state for these analytes, at pH 2.2. For the most efficient separations in capillary electrophoresis, the pH of the run buffer should be close to the pK_a of the analytes. However, in this case only flurazepam has a pK_a close to the pH, and its separation is complicated by ion pairing as will be discussed later. However, it is noticeable that the migration order loosely follows decreasing size of analyte hydrodynamic radius (r), as far as this parameter can be deduced from viewing their structure as in Fig. 2. The electrophoretic mobility, μ_e is related to hydrodynamic radius (r) by Eq. 2:

$$\mu_e = q/6\pi r\eta \quad (2)$$

where q is the charge on the ion and η is the viscosity of the buffer, i.e. the hydrodynamic radius is inversely proportional to electrophoretic mobility. Thus, large molecules of a given charge emerge from the column first and small molecules of the same charge are preferentially retained. To maximise the resolution of peaks I and II, and of peaks III and IV, a separation voltage of −15 kV and a separation temperature of 20°C were used giving rise to R_s values for peaks I and II, and for peaks III and IV, of 2.17 and 1.65, respectively (an R_s value of >1.5 indicates baseline resolution). The addition of organic modifiers, such as methanol or acetonitrile, and variation of temperature had no desirable effect on the separation or peak shape.

Fig. 4a shows the migration behaviour of flurazepam (III) and its metabolites the N^1-(2-hydroxyethyl) analogue of flurazepam (VI) and desalkyl flurazepam (VII). Table 1 shows that, at pH 2.2, flurazepam is protonated at the aliphatic tertiary amine group. The degree of dissociation, α, for the protonated nitrogen in the diazepine group is 0.86. As flurazepam has an overall charge of >1 at pH 2.2, it would be expected to be strongly retained on the capillary column under conditions of reversed polarity. However, it emerges from the column after only 28 min with a much higher efficiency than either of its metabolites. This is believed to be a result of ion pairing of the protonated aliphatic tertiary amine group with $H_2PO_4^-$ anions from the run buffer [12]. Previous capillary electrophoretic migration studies on flurazepam and its metabolites used a citric acid run buffer [13]. At the pH of the run buffer, phosphoric acid exists partly as a singly negatively charged ion, capable of ion pairing,

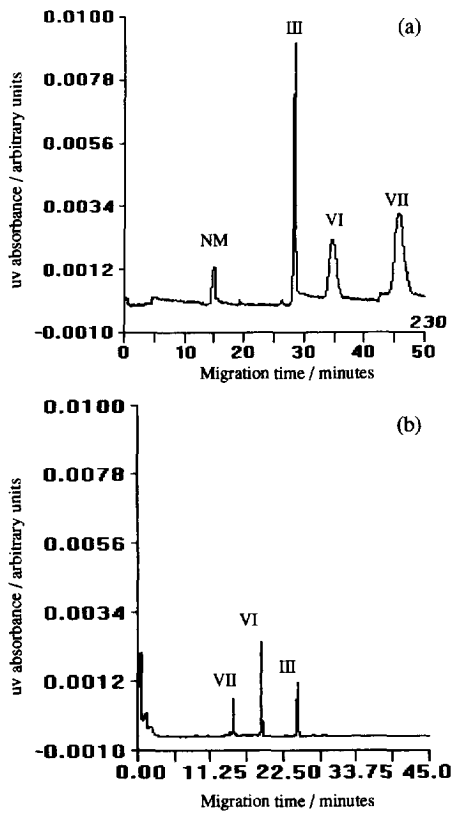

Fig. 4. (a) LVSS–CE electropherogram of flurazepam (III) and its metabolites N^1-(2-hydroxyethyl)flurazepam (VI) and desalkyl flurazepam (VII); (b) CE electropherogram of the same mixture.

Table 1
pK_a data and charge, at pH 2.2, for analytes

Compound	pK_a1	pK_a2	Charge at pH 2.2
Narcotine (I)	9.8[a]	–	+1
Clenbuterol (II)	0.7[a]	9–10[a]	+1
Flurazepam (III)	1.4	8–9[b]	>1
Codeine (IV)	8.2	–	+1
Pethidine (V)	9.8[a]	–	+1
Hydroxyethylflurazepam (VI)	2.26	–	<1
Desalkylflurazepam (VII)	2.56	–	<1

[a] Estimated pK_a.
[b] Estimated using capillary electrophoresis (see Ref. [13]).

whereas citric acid is not dissociated at this pH. Compounds VI and VII have pK_a values of 2.26 and 2.56, giving α values of 0.46 and 0.30 respectively at the pH of the run buffer. As expected, (VI), with its lower pK_a value emerges first from the column followed over 10 min later by (VII), thus underlining the powerful manipulation of separation that can be achieved whenever the pH of the run buffer is close to the pK_a of the analytes. Again, the neutral marker emerges from the column at ca. 14.5 min. All three compounds are completely baseline resolved, having R_s values $\gg 1.5$. Again, the addition of organic modifiers and variation of the temperature did not enhance the resolution or peak shape.

3.2. CE

The separation of the five drugs by conventional CE is shown in Fig. 3b, and, as expected, is the reverse of that for LVSS–CE. The protonated species have an electrophoretic mobility in the cathodic direction which is augmented by the EOF, which is now operating in the same direction so the five drugs migrate faster than in LVSS–CE. The rate of EOF is very slow, with the neutral marker not emerging from the column till after 30 min. Peaks I and II are baseline resolved having an R_s value of 3.31, however peaks III and IV are not baseline resolved, having an R_s value of 1.37.

Fig. 4b shows the electropherogram for the CE separation of flurazepam and its metabolites, again with the expected reverse elution order, and R_s values of $\gg 1.5$.

4. Comparison of LVSS–CE and CE

4.1. LODs

The LOD is defined as being that analyte concentration which gives a signal three times the peak-to-peak noise. From a comparison of the *y*-axes of Fig. 3a and Fig. 3b, and Fig. 4a and Fig. 4b, it is apparent that in each case, large-volume sample stacking greatly increases the amount of analyte that is injected onto the column. The ratio of peak heights for LVSS–CE, compared to CE, ranges from 4–6. This is lower than expected, but can be attributed, in part, to the lower efficiency of LVSS–CE, as will be discussed later. The ratio of peak area for the two modes of capillary electrophoresis gives a much higher value ranging from 20.8 for clenbuterol (II) to 116 for pethidine (V).

Typical LODs for LVSS–CE were of the order of 1×10^{-7} g cm^{-3} compared to a typical value of 1×10^{-6} g cm^{-3} for CE (Table 2). Due to software limitations, the injection volume cannot be increased accurately over 30s. It should be possible to fill the column with a much greater volume of analyte sample and thus to lower LODs even further.

4.2. Efficiency

The migration times of the compounds on the capillary was substantially shorter using CE than LVSS–CE. Efficiencies which are represented by theoretical plates (N) were calculated for both methods. From Table 2, it can be seen that for LVSS–CE, N ranges from 3600 to 89 600 whereas for CE they

Table 2
Comparison of LOD and N values for LVSS–CE and CE

Compound	LVSS–CE		CE	
	LOD/g cm^{-3} ($\times10^{-7}$)	N ($\times10^{4}$)	LOD/g cm^{-3} ($\times10^{-6}$)	N ($\times10^{5}$)
Narcotine (I)	2.40	8.96	1.10	2.25
Clenbuterol (II)	0.92	7.00	0.87	2.11
Flurazepam (III)	1.80	6.86	1.40	1.90
Codeine (IV)	1.08	7.21	0.92	1.85
Pethidine (V)	1.00	4.35	1.3	1.15
Hydroxyethylflurazepam (VI)	5.10	0.46	1.8	0.35
Desalkylflurazepam (VII)	8.20	0.36	1.3	0.47

are in the range 35 000 to 225 000. It is believed that the lower efficiencies for LVSS–CE are responsible for the higher than expected LODs for this technique.

4.3. Reproducibility

A 1×10^{-5}g cm^{-3} solution of flurazepam was injected six times and LVSS–CE separations were carried out. Retention times averaged 28 min with a relative standard deviation (R.S.D.) of 1.64%, and a relative migration R.S.D. of 0.875%. The peak area averaged 354 628 arbitrary units with a R.S.D. of 1.42%.

4.4. Extraction of flurazepam (III) and its metabolites (VI) and (VII) from a urine sample

Extraction of flurazepam and its metabolites was carried out using Toxi-Tubes. Two ml of urine, spiked to a concentration of ca. 1×10^{-5} g cm^{-3} in III, VI and VII, was added to the Toxi-Tube, and the resultant extract was dried and reconstituted in the same volume of water. Fig. 5 shows the resulting electropherogram with a pattern not dissimilar to Fig. 4a. The effective LOD of flurazepam in urine, using this method, was found to be 10^{-6} g cm^{-3}. Whilst this is higher than we would expect to find in clinical investigations, it is relevant to forensic cases, for example, the determination of flurazepam in stomach contents following overdose. Each compound maintained good peak shape and peak areas and the relative migration times were reproducible on repeat injections.

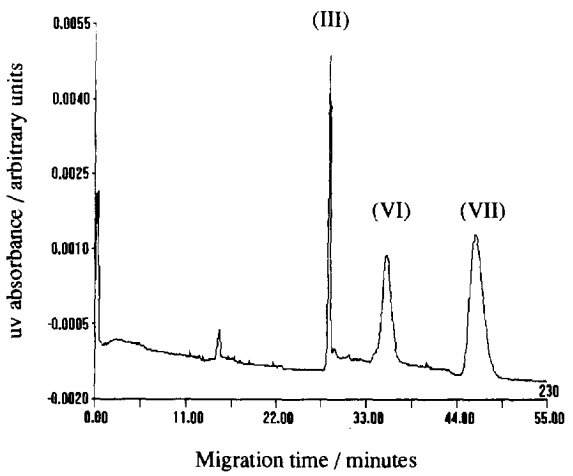

Fig. 5. LVSS–CE electropherogram of a urine sample spiked with compounds III, VI and VII.

5. Conclusion

It has been demonstrated that LODs in CE can be lowered using large-volume sample stacking. Efficiencies for the resulting separations are relatively low compared to conventional CE, resulting in higher than expected LODs for selected drug molecules. By manipulating the software appropriately, it should be possible to lower the LODs to a much greater extent, allowing capillary electrophoretic techniques to gain even more widespread use in trace level forensic and clinical analyses.

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