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The separation and identification of synthetic cathinones by portable low microflow liquid chromatography with dual capillary columns in series and dual wavelength ultraviolet detection

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This study ascertained the viability of a portable liquid chromatograph, operating at low microliter per minute flow, for the analysis of seized drugs at remote sites as well as in laboratory settings. Synthetic cathinones were screened using dual capillary columns in series, C8 and biphenyl, with on-column ultraviolet detection at 255 and 275 nm. The relative retention times of the two columns in series and their peak area absorbance ratio were used to determine if the 16 synthetic cathinones investigated could be uniquely identified in these conditions. Based on these parameters all of the analytes could be distinguished. Representative mixtures of synthetic cathinones were used to determine the repeatability, linearity, and limits of detection of the method. This cost effective and green instrumentation has the potential to satisfy minimum international guidelines for the analysis of seized drugs.

KEYWORDS
dual capillary columns in series, dual on-column UV detection, portable liquid chromatography, synthetic cathinones

1 | INTRODUCTION

There is a demand for portable on-site and laboratory instrumentation that can detect and identify drug evidence. These instruments are generally light weight and can be easily moved throughout a laboratory and brought to the crime scene. Most of these portable techniques are based on Raman and IR spectroscopy [1–5]. Portable Raman spectroscopy is valuable for on-site detection of drugs because it does not need to be in contact with the substance and can even detect drugs through plastic bags, which is very common packaging for illicit substances [4]. The spectroscopic techniques are advantageous for forensic purposes because they are rapid and nondestructive. However, IR and Raman spectra can become too complex to interpret in the case of mixtures. Other portable techniques, such as portable microchip electrophoresis [6] and portable mass spectrometers equipped with desorption electrospray ionization (DESI), have the ability to detect and discriminate between seized drugs [7]. The portable microchip electrophoresis with laser emitting diode induced fluorescence technique requires derivatization and uses only migration times for identification purposes [6]. While the portable mass spectrometer is viable

Article Related Abbreviations: BEH, ethylene bridged hybrid; DART, direct analysis in real time; EI, electron ionization; IMS, ion mobility spectrometry; LED, light emitting diode; PDA, photodiode array; PFP, pentaflurophenyl; RRT, relative retention time; SWGDRUG, the scientific working group for the analysis of seized drugs; UHPSFC, ultra high performance supercritical fluid chromatography

for mixtures, it produces only molecular ion information, which is particularly limited in screening for positional isomers, isobars, and stereoisomers.

The Scientific Working Group for the Analysis of Seized Drugs (SWGDRUG) developed the minimum standards for the forensic identification of seized drug evidence [8]. According to SWGDRUG, the identification of a drug depends on the analytical method and the competence of the analyst. It requires that multiple uncorrelated techniques be used to properly identify seized drugs [8]. There are three categories of techniques (A, B, and C) that can be used to identify seized drugs, with A being the most discriminating and C being the least discriminating. Category A techniques, including MS, offer insight into the molecular structure of the seized drugs; Category B techniques, including LC and UV spectroscopy, measure specific chemical or physical characteristics of the seized drugs; and Category C techniques, including color tests, provide nonspecific chemical or physical characteristics [8]. The minimum requirements for the identification of a seized drug include one Category A test with at least one other technique from any of the three categories. When a Category A test is unavailable, or one chooses not to use this category, at least three separate techniques need to be used, with at least two from Category B [8].

Synthetic drugs produced in clandestine laboratories have recently become a large source of abused substances [9]. These substances, known as designer or emerging drugs, offer the same effects of illicit drugs while avoiding the penalties of controlled substances laws [9–13]. The emerging drugs are sold as "legal highs" under unique and appealing names [9,10,12, 13]. Synthetic cathinones are one class of emerging drugs [10–12]. Synthetic cathinones are a group of β-ketone phenethylamine compounds synthesized to mimic the active stimulant of the khat plant, cathinone [10,13]. Some street names for these designer drugs include meow meow, bliss, white rush, blue magic, blue silk, cloud 9, and rocket fuel [13]. Synthetic cathinones are sold under discrete names, such as bath salts, plant food, plant feeders, fertilizer, insect repellents, and research chemicals, and can be purchased online, at smoke shops, and at convenience stores [10,12,13]. These drugs are typically white to light brown powders or crystals and produce the same effect in users as stimulants such as methamphetamine, methylenedioxymethamphetamine (MDMA), and cocaine [10–13]. To reduce the access to and consumption of synthetic cathinones, they were classified as Schedule I drugs after a large number of overdoses [10].

Additional tests to those recommended by SWGDRUG are carried out by forensic laboratories in order to increase specificity of analysis. This is particularly important when analyzing emerging drugs which, in a given class, contain similar compounds such as analogues, positional isomers, and diastereomers. In addition, complementary procedures would mitigate the mixing of samples.

There are several methods that can aid in the identification of synthetic cathinones including colorimetric detection, immunochemical detection, and instrumental detection. Color tests are employed as a presumptive test because they are rapid, inexpensive, and simple, however, they are highly susceptible to false positives. Currently, there are no specific color tests for any individual synthetic cathinone. One reagent—copper(II)-2,9-dimethyl-1,10-phenanthroline (Cu(II)-neocuproine)—has been tested for the detection of synthetic cathinones [14]. This spot test turns yellow orange when reacted with synthetic cathinones and yielded a positive result for 39 of the 44 synthetic cathinones tested [14]. Immunoassays are other presumptive tests for the detection of certain seized drugs because they are rapid and inexpensive, however, they are subjected to cross-reactivity of nontargeted compounds. These tests use antibodies to detect the presence of drugs. One study evaluated synthetic cathinones, as well as other designer drugs, against 16 different commercial ELISA reagents to determine cross-reactivity [15]. The Randox mephedrone/methcathinone kit was the only immunoassay that could detect a wide range of synthetic cathinones [15]. Numerous instrumental methods can be used to detect and identify synthetic cathinones, including GC-EI-MS [16-21], GC-EI-MS/MS [16,22,23], gas chromatography cold electron impact with quadrupole time-of-flight mass spectrometry (GC-cold EI-QTOF-MS) [17], gas chromatography with positive chemical ionization tandem mass spectrometry (GC-PCI-MS/MS) [22], gas chromatography infrared spectroscopy (GC-IR) [16], gas chromatography with vacuum ultraviolet detection (GC-VUV) [24], high performance liquid chromatography with photodiode array ultraviolet detection (HPLC-UV PDA) [23], ultra high performance liquid chromatography with electrospray time of flight mass spectrometry (UHPLC-ESI-TOF-MS) [19,25], UHPLC-UV PDA-ESI-MS [26,27], UHPLC-ESI-MS/MS [28], multidimensional UHPLC-UV PDA-ESI-MS [29], capillary electrophoresis (CE-UV PDA) [27], CE-UV PDA-MS [30], ultrahigh performance supercritical fluid chromatography (UHPSFC)-UV PDA-ESI-MS [11,19,31], ion mobility spectrometry (IMS) [32], IMS-ESI-MS [32], direct analysis in real time (DART)-TOF-MS [33], DART-IMS-TOF-MS [34], Raman spectroscopy [35], portable Raman spectroscopy [1–5], IR spectroscopy [35], portable IR spectroscopy [1-5], and nuclear magnetic resonance spectroscopy (NMR) [36,37]. These instruments either lack portability, and/or are lacking in specificity, and/or are problematic for mixtures.

Miniaturized separation techniques, including CE, capillary electrochromatography, and capillary/micro/nano-LC, have become more popular for drug analysis in the

last decade [38]. The advantages of these techniques are relatively high separation efficiency and resolution, rapid analysis, and minimal consumption of reagents and samples [38,39]. Very few studies have employed capillary LC for the analysis of seized drugs [39]. Until recently, miniaturized LC separation instrumentation was not portable. Abonamah et al [40] used a field "portable" nano-LC with EI-MS for the onsite detection of fentanyl and fentanyl derivatives. An Acclaim Pepmap 100 C18, 3 μm, 0.075 × 150 mm column was used, with separations performed at 0.1-1.0 μL/min. However, the LC instrumentation alone was 37 kg and required a generator to operate. The instrumentation could be taken into the field but was not considered portable like previously mentioned IR and Raman spectroscopy instrumentation. A truly portable miniaturized LC system, with on-column light-emitting diode (LED)-based UV-absorption detection (260 nm) that is light-weight (5.9 kg), has low mobile phase consumption and waste generation, and can be battery operated was developed [41,42]. A packed capillary column (Acquity BEH C18, 1.7 μm, 100 mm × 150 μm i.d) was employed with separations performed at a flow rate of 2 μL/min. The LED detector was designed and optimized for on-column detection to minimize extra-column band broadening [42]. The coupling of the portable LC to an orbitrap MS was also investigated [41]. Xie et al [43], employing a single capillary (10 cm x 150 μm i.d. with 3 μm C18 particles), used miniaturized LED-based UV-absorption detectors for on-column low microflow (1.5 μL/min) LC at two wavelengths, 255 and 275 nm, for the separation and detection of a phenol mixture. It demonstrated the potential of using absorbance ratio measurements for target analyte detection [43]. A further study using this instrumentation (C18 2 μm, 100 mm × 150 μm with single on-column LED UV) investigated its applicability for pharmaceutical and illicit drug analysis [44]. Examples of the separation of two to five drugs (including metabolites) for a few classes of seized drugs is given. Foster et al stated that one challenge of this instrumentation applied to drug analysis exists because of the vast array of molar absorptivity values of different classes of drugs [44], which can lead to lower limits of detection for compounds that do not absorb strongly at a given LED wavelength.

The use of relative retention times (RRTs) and dual wavelength absorbance ratios (proportional to peak area ratios) to identify drugs was first demonstrated by Baker et al [45]. This study analyzed and characterized 101 drugs by HPLC with UV detection at 245 and 280 nm. Baker et al demonstrated that only 9% of drugs could be distinguished by RRTs alone (using a single column) but with the addition of absorbance ratios, 95% of the drugs could be distinguished [45]. A later study proved that 26 fentanyl analogues and homologues could also be distinguished when combining RRT with UV detection at 215 and 230 nm [46].

To demonstrate the utility of the portable low microflow LC for seized drugs, the present study employs two capillary columns in series, that is, C8 and biphenyl, with dual on column UV detection, that is, 255 nm and 275 nm, for the screening and/or identification of different synthetic cathinones.

2 | MATERIALS AND METHODS

2.1 | Materials and reagents

The 16 synthetic cathinones used in this study are shown in Fig. 1. These standards were purchased from Cayman Chemical (Ann Arbor, MI, USA). Optima® LC/MS Grade acetonitrile, Optima® LC/MS Grade water, and Optima® LC/MS Grade trifluoroacetic acid (TFA) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). MicroSolv AQ™ Nylon 0.2 μm syringe filters were acquired from MicroSolv Technology (Leland, NC, USA).

2.2 | Preparation of solutions

Mobile phase solvents were premixed as suggested by the portable LC vendor. Solvent A was 94.9% water and 5% acetonitrile with 0.1% TFA, while Solvent B was 4.9% water and 95% acetonitrile with 0.1% TFA. Synthetic cathinone samples (prepared from methanol stock solutions) were brought to dryness and diluted with Solvent A at a concentration of 33 ppm unless otherwise stated. For the linearity study, serial dilutions of synthetic cathinones were prepared from 500 to 3.9 ppm.

All synthetic cathinone solutions were centrifuged for at least 10 min prior to injection. In addition, all mobile phase solvents were filtered with MicroSolv AQ™ nylon 0.2 μm syringe filters.

2.3 | Instrumentation

The Axcend Focus LC system (Provo, Utah, USA) fitted with dual syringe pumps to provide mobile phase gradient capability, pressures up to 10 000 psi, and a 500 nL external injection loop, was used for the analysis of all samples. The preliminary experiments to determine the feasibility of using this portable low microflow LC with dual UV detection for synthetic cathinones utilized an Axcend Focus LC Cartridge that contained a 3 μm, 0.15 × 100 mm C18 column with on-column dual UV LED detection at 255 and 275 nm. All other experiments utilized an Axcend

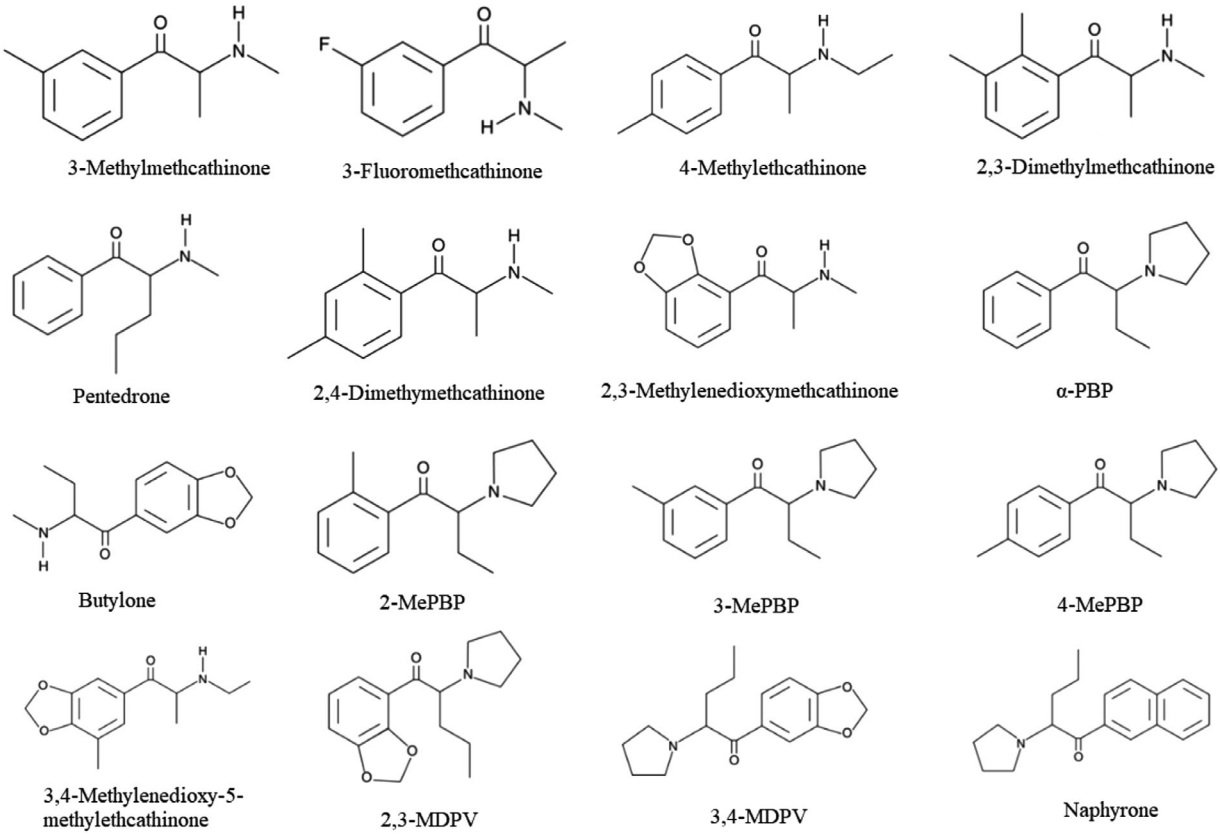

FIGURE 1 Synthetic cathinones analyzed

Focus LC Cartridge that contained a 3 μm, 0.15 × 100 mm C8 column in series with a 3 μm, 0.15 × 50 mm biphenyl column with dual on-column UV LED detection at 255 and 275 nm. The instrument was controlled by the Axcend Drive software available on a laptop computer connected to the instrument through an ethernet cable.

2.4 | Portable LC method

The conditions utilized for determining the specificity of analysis for the synthetic cathinones, using the dual capillary cartridge were as follows: 5 μL manual injection (overfill 500 nL injection loop), ambient temperature, 3 μL/min flow rate, and 5 min pre-equilibration at initial gradient conditions. The initial mobile phase conditions were 95% A, 5% B and the final conditions were 75% A, 25% B. The gradient was linear for 10 min with a 3 min hold at the final conditions.

2.5 | Figures of merit

The uniqueness of the RRTs and peak absorbance ratios were determined by two times the standard deviation for an average of five runs. The percent relative standard deviation (%RSD) was also calculated to compare the uncertainty between repeat measurements. These calculations were carried out with Microsoft Excel (Redmond, Washington, USA). The LOD was calculated by manually measuring both the height of the analyte signal peaks at a low concentration (from a serial dilution) and the height of the noise peaks. The signal to noise ratio was calculated at the measured concentration, and the desired LOD value (two times signal-to-noise) was extrapolated from this measurement.

3 | RESULTS AND DISCUSSION

Preliminary work was conducted using a single capillary column with dual UV detection at 255 and 275 nm for the separation of synthetic cathinones. The capillary LC system was found suitable for the analysis of this class of emerging drugs based on chromatographic performance and the responses at both wavelengths for a select number of synthetic cathinones, the latter which were consistent with known UV spectra of these analytes [26].

Sixteen synthetic cathinones were analyzed by the portable low microflow LC using a RP gradient with dual

TABLE 1 Average relative retention times and peak area ratios, standard deviation, and percent relative standard deviation of 16 synthetic cathinones at 33 ppm run five times

Compound	C8 RRT	2σ	%RSD	Biphenyl RRT	2σ	%RSD	Peak area ratio (255/275)	2σ	%RSD
3-Fluoromethcathinone	0.181	0.001	0.495	0.240	0.003	1.101	5.14	0.141	2.743
2,3-methylenedioxymethcathinone	0.185	0.000	0.252	0.251	0.002	0.842	2.80	0.035	1.252
3-methylmethcathinone	0.221	0.001	0.458	0.344	0.002	0.661	3.38	0.088	2.595
α-PBP	0.249	0.002	0.705	0.359	0.003	0.849	4.13	0.061	1.480
4-methylethcathinone	0.251	0.001	0.452	0.390	0.001	0.359	1.29	0.008	0.630
Pentedrone	0.280	0.003	0.899	0.433	0.003	0.703	4.95	0.042	0.845
Butylone	0.299	0.005	1.682	0.343	0.014	3.972	0.26	0.003	1.040
3,4-methylenedioxy-5-methcathinone	0.318	0.003	0.962	0.458	0.003	0.716	0.17	0.010	5.950
2,4-dimethylmethcathinone	0.327	0.002	0.647	0.501	0.002	0.362	1.55	0.006	0.412
2,3-dimethylmethcathinone	0.336	0.010	3.061	0.496	0.009	1.820	3.19	0.074	2.312
2-MePBP	0.365	0.004	1.230	0.524	0.003	0.593	4.56	0.071	1.556
4-MePBP	0.392	0.005	1.208	0.545	0.003	0.515	1.09	0.007	0.686
3,4-MDPV	0.426	0.003	0.796	0.568	0.002	0.289	0.27	0.018	6.767
3-MePBP	0.430	0.012	2.814	0.571	0.007	1.160	2.90	0.150	5.182
2,3-MDPV	0.430	0.004	0.885	0.575	0.003	0.494	1.84	0.032	1.719
Naphyrone	1.000	0.000	0.000	1.000	0.000	0.000	4.95	0.055	1.102

columns in series, C8 and biphenyl, with dual on-column wavelength detection at 255 and 275 nm, respectively. RRTs for both stationary phases and peak area ratios are given in Table 1.

To determine whether a combination of dual RRTs and peak area ratios at both wavelengths could uniquely identify each target compound, repeatability studies were performed. The RRTs and peak area absorbance ratios shown in Table 1 were calculated from an average of five runs. For both RRTs (relative to naphyrone) and absorbance ratios, two times the standard deviation (2σ) was calculated to determine the uniqueness of these individual values. Based on these values, 56% (9/16) of synthetic cathinones can be distinguished by the RRT of the first column, 69% (11/16) of synthetic cathinones can be distinguished by RRTs of the two columns, and 100% (16/16) of synthetic cathinones can be distinguished by both RRTs and their peak area UV absorbance ratio.

Included in this study were three sets of positional isomers with mass 191 (pentedrone, 4-methylethcathinone, 2,3-dimethylmethcathinone, and 2,4-dimethylmethcathinone), mass 231 (2-MePBP, 3-MePBP, and 4-MePBP), and mass 275 (2,3-MDPV and 3,4-MDPV). All positional isomers were distinguished (Table 1) based on dual RRTs and UV absorbance ratios.

Clearly, the use of absorbance ratios significantly improved the specificity of analysis over the use of dual RRTs. In fact, all 16 synthetic cathinones could be distinguished based on RRTs of the first column and absorbance ratios. It should be noted that there could be a small spectral shift for peaks eluting in the second capillary versus the first capillary, due to a change in the ratio of organic to aqueous in the mobile phase, which would affect the dielectric constant [11]. According to SWGDRUG guidelines, two orthogonal Category B separation techniques in addition to another Category B or C technique could be employed for the identification of seized drugs [8]. Further, according to these guidelines, a high degree of selectivity is achieved when the two chosen Category B techniques exploit different chemical or physical properties of the analytes, that is, separations employing different interactions of the analyte with the stationary phase. For RP chromatography, these interactions include hydrophobic, steric, hydrogen bonding, dipole, ion exchange, and π interactions [47]. For a study examining multiple stationary phases, which can explore the different interactions, 11 selectivity differences were measured by comparing S^2 values which are highly dependent on R^2 values, that represent the correlation of the retention times between two stationary phases [47]. Since $S^2 = 1 - R^2$, the greater the R^2 value the smaller S^2. Gilar et al [48] for comprehensive 2D LC stated that if the data points only covered 10% of a plot area, which is similar to a straight diagonal line with an R^2 value of 1, then there is 0% orthogonality. Therefore, the greater the selectivity differences of the stationary phases employed for 2D LC, the greater the coverage of the plot area. Ochoa et al [29] for heart cutting 2D LC showed that the fractional coverage in 2D LC

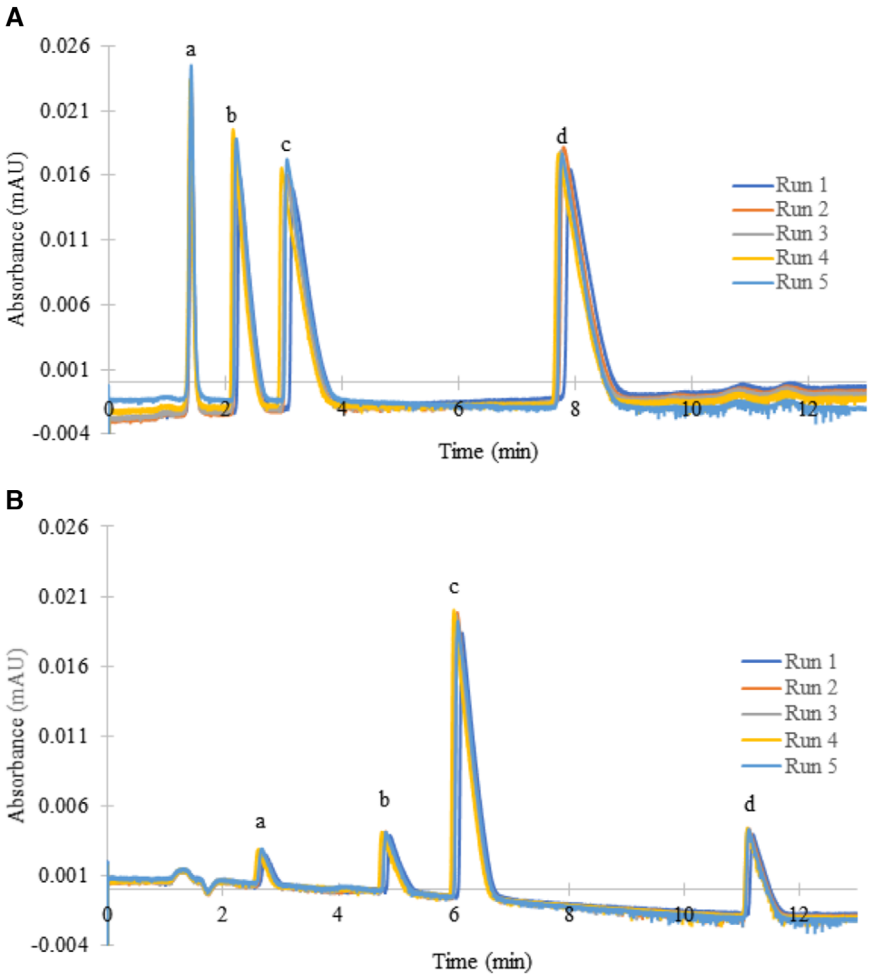

FIGURE 2 Overlay of five runs of a representative synthetic cathinone mixture prepared at 33 ppm (a) 3-fluoromethcathinone (b) pentedrone (c) 4-MePBP (d) naphyrone at (A) C8 and 255 nm and (B) biphenyl and 275 nm

can be approximated by S^2, as the increased selectivity differences between columns indicates that more of the separation space is used. The C8 and biphenyl columns used in the present study, for which no solvent modulation is employed, exhibited an R^2 value of 0.9322 which showed little selectivity differences between the stationary phases employed. In order to obtain a greater degree of selectivity, that is, considerably lower R^2 values, and therefore, better meet SWGDRUG guidelines, alternative stationary phase combinations could be investigated. For RP, the use of an unconventional pentaflurophenyl (PFP) column, which exhibits unique interactions due to presence of fluorene groups on the phenyl ring, would be an interesting choice [47]. The use of silica hydride stationary phases, which exhibit unique selectivity compared to conventional silica columns, could provide enhanced orthogonality [49]. The use of different stationary phases in the hydrophilic interaction chromatography (HILIC) or aqueous normal phase mode, would be interesting to explore [49,50]. It would appear that the use of absorbance ratios, which exhibited a relatively high degree of specificity, would qualify at least as a Category C technique.

Taking into account that the instrument does not require a major capital expenditure, only weighs 8 kg, is extremely green (using approximately 1.4 mL of mobile phase per day with low waste generation), and can operate at up to 10 h on a rechargeable battery, the investigative technology is not only amenable for drug screening at remote sites, but well suited as a complementary method or possibly primary method for drug identification, particularly for developing countries.

For most synthetic cathinones, the %RSD of the repeatability of RRT for both stationary phases were ≤1.2, while the %RSD of repeatability of peak area ratios were ≤2.7 (Table 1). An example of an overlay of five injections for one of the representative synthetic cathinones mixtures is shown in Fig. 2. The LC system employed in this study generates flow using a calibrated constant displacement of the syringe pumps and mixing the mobile phases in the solvent valve. There is no flow meter in the pumping system that provides feedback to the pumps regarding the actual flow. Due to viscosity changes, flow may vary over the run. Adding a flow controller to the pumping system for more precise flow control would result in more repeatable

TABLE 2 LOD (2 × signal-to-noise) and determination coefficients of synthetic cathinones at 255 and 275 nm

Synthetic Cathinone	LOD 255 nm (ppm)	LOD 275 nm (ppm)	255 plot range	R^2	275 plot range	R^2
3-Fluoromethcathinone	0.87	5.22	7.18-500	0.998	31.2-500	0.9996
α-PBP	1.12	2.40	7.18-125	0.9992	15.6-500	0.9992
4-MePBP	1.30	0.64	7.18-500	0.9997	3.91-500	0.9997
Naphyrone	0.89	1.64	7.18-500	0.9995	15.6-500	0.9993
2,3-Methylenedioxymethcathinone	0.39	1.30	3.91-250	0.9968	7.18-250	0.9969
Pentedrone	0.82	2.08	7.18-125	0.9991	15.6-250	0.9962
2,4-Dimethylmethcathinone	2.04	2.89	15.6-500	0.9991	15.6-500	0.9975
3-Methylmethcathinone	1.87	1.30	15.6-500	0.9976	7.18-500	0.9972
2-MePBP	2.49	3.26	15.6-500	0.9996	31.2-500	0.9998
2,3-MDPV	2.76	2.00	15.6-500	0.9992	15.6-500	0.9992

retention times and peak areas. In addition, the most recent software (which was not used in this study) enables the user to run under active pressure-control. In this case, the flow is changing, and pressure is constant during the run. Gritti et al [51] showed advantages for operating at the maximum pressure (constant pressure mode) during the gradient run at operating pressures less than 250 bar. For a 5-95% methanol gradient, besides compensating in changes in viscosity during the run, operating at constant pressure under these conditions provides for a faster analysis (about 20% shorter) without loss in peak capacity. For the present study, although operating at higher pressures (>250 bar) could affect both retention times and RRTs, for a maximum pressure of 470 bar there was only an approximate 10% change in pressure during the run. Therefore, similar peak capacities and run times should be expected when operating in the constant pressure mode.

The LOD for representative synthetic cathinones ranged from 0.39 to 12.5 ppm at 255 nm and 0.64 to 5.22 ppm at 275 nm (Table 2). For these same analytes, the linearity of the synthetic cathinones for both UV wavelengths spanned at least one order of magnitude and had a determination coefficient of 0.996 or greater (Table 2). This indicates the utility of the proposed methodology for quantitative analysis.

4 | CONCLUDING REMARKS

The low weight of the instrument (only 17.6 pounds or 8 kg), low solvent usage, low waste production, 10+ h battery life, and built in Wi-Fi allows for easy portability. In addition, this capillary LC system exhibits high specificity and ability to separate mixtures. The 16 synthetic cathinones analyzed in this study (including three sets of positional isomers) were uniquely identified by both RRTs of the dual columns in series and the UV peak area ratios. Therefore, the portable liquid chromatograph is well suited for both laboratory and remote settings. To meet SWGDRUG requirements as a stand-alone instrument, a different combination of stationary phases that produce orthogonal retention times would be required.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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