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Guilty by dissociation: Part C: Enantiomeric separation of diphenidine-derived new psychoactive substances (NPS) by polar organic chiral high performance liquid chromatography (HPLC) on polysaccharide-based stationary phases

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ABSTRACT

This study describes a simple and practical HPLC analysis for the direct enantiomeric separation of a range of 32 novel diphenidine derived psychoactive substances using a range of six polysaccharide-based chiral stationary phases employing a single generic polar organic solvent chromatographic mobile phase. Temperature was employed to optimize the chemo and enantiomeric selectivity. Baseline separation and differentiation of both the enantiomers and positional isomers (i.e., regioisomers) of the 2-, 3- and 4-methoxphenidines was achieved with the chiral selector cellulose *tris*(3-chloro-4-methylphenylcarbamate) coated onto silica. The latter proved to be the best of the six chiral stationary phases investigated in that it generated enantiomeric separation of 25 of the 26 monosubstituted diphenidines with resolution values > 1.5. It yielded the optimum separation for 21 of the 26 diphenidines (resolution values ranged from 2.9 – 22.4) including the 2-, 3- and 4-positional isomers of eight diphenidine derivatives. Excellent separation of all 26 monosubstituted diphenidines (i.e., resolution values > 1.5) and peak shape (i.e., typical tailing factors between 0.9 – 1.2) could be achieved by using Lux Cellulose-2 and Lux *i*-Amylose-3 columns. The nature of the polysaccharide-based chiral selector was demonstrated to be extremely important in determining the degree of chiral resolution. The location of the monosubstituent on the 1-phenyl ring of the diphenidine was shown to be important in promoting chiral resolution. Greater chiral discrimination was typically observed for substituents in the 4-position compared to those in the 2-position of the 1-phenyl ring. The chiral HPLC methodology displayed good chemo and enantiomeric selectivity of the mono-, di- and trisubstituted diphenidine regioisomers. Enantiomer elution order reversal was highlighted with 2-methoxphenidine enantiomers as a function of the chiral stationary phase. The (R)-enantiomer eluted before the (S)-enantiomer on cellulose-based chiral stationary phase whereas the reverse occurred with the amylose-based phases. Application of the methodology to the analysis of real-life samples of 2-methoxphenidine and diphenidine confirmed that these psychoactive substances were being traded as racemic products. Commonly used adulterants in powdered samples were shown not to interfere with the chiral analysis of 2-methoxphenidine and diphenidine.

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1. Introduction

Over the past fifteen years, there has been a significant increase in the number of novel (or new) psychoactive substances (NPS) seized by law enforcement agencies globally [1]. NPS are materials in their pure form, or in a preparation, that are not covered by the United Nations Single Convention on Narcotic Drugs (1961), as amended by the Protocol (1972), or by the United Nations Convention on Psychotropic Substances (1971) but can potentially lead to adverse health or social risks similar to those posed by the substances covered by the conventions [2]. Within this context, the terms "novel" or "new" does not necessarily refer to novel inventions but to substances that have recently become available on the illicit market. Psychoactive substances prohibited under the international drug control conventions produce their effects through a small number of pharmacological mechanisms and can have significant chemical diversity within each family of psychoactive substances [1,2]. Current convention uses a functional "effect group" categorization to define NPS within six broad overlapping groups: (i) cannabinoid receptor agonists; (ii) classic hallucinogens; (iii) dissociatives; (iv) opioids; (v) sedatives/hypnotics and (vi) psychostimulants. The grouping is based on the features related to their chemical structure, psychopharmacological desired and unwanted effects [1,2].

1,2-Diarylethylamines (or diphenidines) are dissociative, psychoactive substances which distort perceptions, produce feelings of detachment, and induce a state of anaesthesia by antagonizing ionotropic *N*-methyl-D-aspartate receptors (NMDAR) in the central nervous system [2,3]. The first of these dissociative anaesthetics was 1-(1, 2-diphenylethyl)piperidine (diphenidine, **1**) [3], followed by 1-[1-(2-methoxyphenyl)-2-phenylethyl]piperidine (2-methoxphenidine, 2-MXP, **2a**) [4] which have both been marketed as "research chemicals" and encountered in both tablet or powder forms [3–6] or in combination with synthetic cannabinoid receptor agonists [7]. Though both the supply and production of **1** and **2a** and the recently disclosed 1-[1-(2-chlorophenyl)-2-phenylethyl]piperidine (2-chlorodiphenidine, 2-Cl-DPH, **5a**) [8] are now controlled in the United Kingdom by the Psychoactive Substances Act (2016) [9], the emergence of novel 1,2-diarylethylamine derivatives, such as **5a**, still raises considerable legal and analytical challenges in forensic identification, due to their inference in several fatalities in Europe [10] and Asia [7] and on 14th April 2021, diphenidine was placed under international control, within schedule II of the

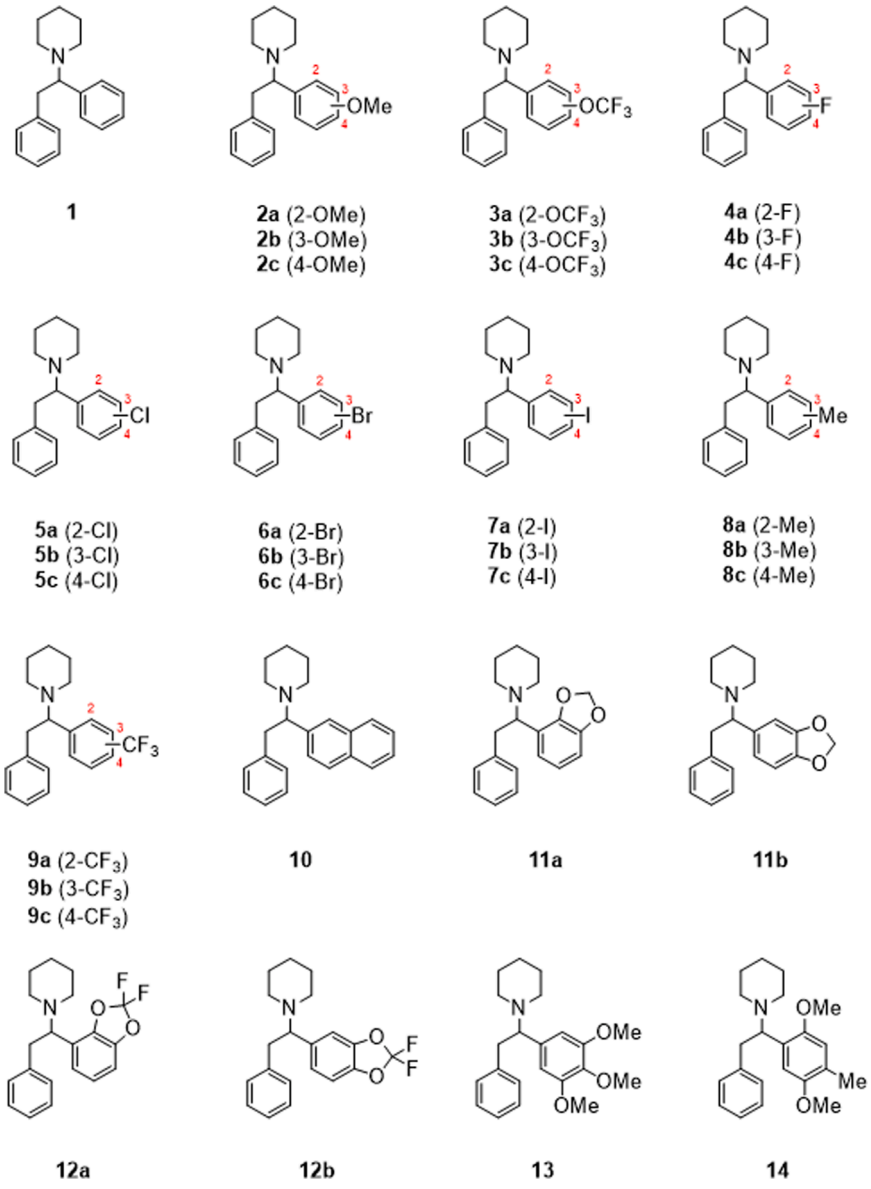

Fig. 1. Structures of the regioisomeric diphenidines (**1–14**) utilized in this study.

United Nations Convention on Psychotropic Substances (1971) [11].

1,2-Diarylethylamines possess one chiral centre and therefore exist as two stereoisomers [1,3] and although it is believed that these compounds are marketed as racemic mixtures, there appears to be little information on the enantiomeric separation of bulk samples. Pharmacological studies have reported large differences in NMDAR affinity between the two enantiomers of diphenidine: (+)-(S)-**1** showed a 40-times higher affinity than the (−)-(R)-**1** enantiomer ($K_i = 130$ nM *vs.* 5.25 nM respectively) [12], however there is a paucity of information regarding the NMDAR affinity for other 1,2-diarylethylamine derivatives. Assuming similar pronounced differences, the development of chiral separation methods for these compounds is vital to understand the structure-activity relationships for these psychoactive substances.

As a continuation of our research into the High Performance Liquid Chromatography (HPLC) and Superficial Fluid Chromatography (SFC) analysis of NPS (see Parts A and B respectively [6,13]), direct chiral HPLC was employed to evaluate the enantiomeric resolution of the racemic diphenidine derivatives possessing either electron donating or withdrawing substituents of varying size and lipophilicity on the 1-phenyl ring (see Fig. 1) [5,6,13]. These included the 2-, 3- and 4-positional isomers (commonly known as the *ortho*-, *meta*- and *para*--regioisomers) of eight diphenidine families (**2a–2c**, **3a– 3c**, **4a–4c**, **5a–5c**, **6a–6c**, **7a–7c**, **8a–8c**, and **9a–9c**) and two groups of twinned structural isomers (**11a**/**11b**, and **12a**/**12b**) [6,13,14].

Compared to their achiral analysis, there has been limited research into the enantiomeric separation of NPS. Various chromatographic and electrophoretic techniques such as HPLC [15–19], capillary electrophoresis (CE) [20,21] and gas chromatography (GC) [22,23], supercritical fluid chromatography (SFC) [13,19] and capillary electrochromatography (CEC) [19] have been reported to provide enantiomeric separation of certain NPS.

Diphenidine (**1**) and 2-methoxphenidine (**2a**) [15,16] and a range of other NPS have previously been reported to be resolved by HPLC using a polysaccharide-based chiral stationary phase (CSP) employed in the polar organic solvent chromatography (POSC) mode using mobile phase comprising of acetonitrile:2-propanol:diethylamine:formic acid (95:5:0.1:0.1 v/v/v/v). Chiral HPLC conditions such as these are popular in many generic chiral screening approaches [16,24–28]. Due to the lack of chiral methodologies for the enantiomeric separation of the forensically important diphenidines an investigation was undertaken into the HPLC operating parameters affecting their separation on six differing CSP (i.e., differing chiral selectors (CS) based on cellulose or amylose, coated or immobilized onto chromatographic silica) using POSC conditions. This was then extended to additional novel diphenidines which have not yet been used as illicit NPS.

The optimized chiral HPLC conditions were then applied to the analysis of eight seized bulk samples of (**1**) and (**2a**) to assess their enantiomeric purity and to ascertain whether the compounds are traded as pure enantiomers, racemic mixtures or possibly adulterated with other drugs of abuse.

2. Materials and methods

2.1. Solvents and diphenidine standards and procurement of forensic samples

Acetonitrile (ACN), 2-propanol (IPA), methanol (MeOH) and ethanol (EtOH) were of HPLC grade and supplied by Romil Limited (Cambridge, UK). Formic acid (FA) and diethylamine (DEA) were of HPLC grade and supplied by Sigma Aldrich (Poole, UK). Diphenidine (**1**) and its derivatives (**2a**–**14**, Fig. 1) were prepared by MANchester DRug Analysis and Knowledge Exchange (MANDRAKE). The synthesis of the racemic target compounds was achieved using the previously reported method [5,6,13] and isolated as their corresponding hydrochloride salts. To ensure the authenticity of the reference materials utilized within this study, the 32 synthesized samples were structurally characterized by ^{1}H NMR, ^{13}C{^{1}H}-NMR, GC-MS and ATR-FTIR and the purity of all samples was confirmed to be > 99.5 % (by NMR) in all cases. The NMR purity was calculated, as previously described using the relative concentration determination method [5,6,13]. Eight seized diphenidine samples were provided to MANDRAKE, between June – October 2016 and October – December 2023, by Greater Manchester Police, in accordance with Manchester Metropolitan University's Home Office license requirements and agreed procedures. To ensure the authenticity of the seized bulk samples, utilized within this study, the principal components and purity was confirmed to be > 98.7 % (by GC-MS) in all cases [5].

2.2. High performance liquid chromatography (HPLC)

HPLC-PDA analysis was performed on a Shimadzu Nexera XS UHPLC (Shimadzu UK Ltd, Milton Keynes, UK) equipped with two binary pumps (LC-40D XS) and proportioning valves, degassers (DGU-40S), autosampler with cooling capabilities (SIL-40C XS), column oven (CTO-40C), diode array detector (SPD-M30A) with a 1 μL / 10 mm pathlength flow cell, 180 μL mixer and communication bus module (CBM-40Lite). The system was controlled, and data collected by means of Shimadzu LabSolutions software (version 5.86).

2.2.1. Sample preparation

Stock solutions of the diphenidines (**1–14**) were prepared at a concentration of 1 mg/mL in ACN.

2.3. Generic chiral high performance liquid chromatography-photodiode array spectrometry chromatographic conditions

Before use, each new chiral column was flushed with at least 20 column volumes as follows: 9:1 v/v MeOH:EtOH to displace the mobile phase in which the columns had been supplied and then with 95:5:0.1:0.1 v/v/v/v ACN:IPA:FA:DEA for adequate equilibration. After use and before being stored, columns were flushed with at least 20 column volumes of 9:1 v/v MeOH:EtOH. Each time the mobile phase was changed the flow rate was increased using a linear ramp from 0.0 to 1 mL/min over 5 min. Unless otherwise stated, the following HPLC conditions were employed: a flow rate of 1 mL/min, column oven and autosampler temperature of 20 and 10 °C respectively, 5 μL injection volume. Chiral HPLC was performed on 150 × 4.6 mm I.D. columns supplied by Phenomenex (Torrance, CA, USA) as shown in Table 1. The chiral packing materials were made with polysaccharide-based chiral selectors and 5 μm surface-modified silica particles. The integrity of all the columns was confirmed periodically throughout the experiments by injecting racemic 2-methoxphenidine (**2a**) before and after the experiments. All columns maintained retention times, efficiency and peak symmetry levels of > 95 % of their initial value. The first baseline disturbance for a ACN injection was used as the dead time marker. The photodiode array (PDA) detector was set to monitor each diphenidine at their optimum wavelength (Supplementary Material 1) (bandwidth 8 nm) with a reference set at 360 nm (bandwidth 100 nm). The data sampling rate was set at 12.5 Hz giving typical peak widths of 1 - 2 min. Chromatographic values reported are the average of duplicate injections.

Table 1
Chiral stationary phases.

Column name	Ligand
Lux Cellulose−1	Coated Cellulose *tris*(3,5-dimethylphenylcarbamate)
Lux Cellulose−2	Coated Cellulose *tris*(3-chloro−4-methylphenylcarbamate)
Lux Cellulose−3	Coated Cellulose *tris*(4-methylbenzoate)
Lux *i*-Cellulose−5	Immobilized Cellulose *tris*(3,5-dichlorophenylcarbamate)
Lux *i*-Amylose−1	Immobilized Amylose *tris*(3,5-dimethylphenylcarbamate)
Lux *i*-Amylose−3	Immobilized Amylose *tris*(3-chloro−5-methylphenylcarbamate)

2.3.1. Chiral separation of 2-, 3- and 4-methoxphenidine (2a-2c) on Lux Cellulose-2 CSP as a function of temperature

The HPLC conditions as described in Section 2.3 were employed using a column oven temperature ranging between 15 and 30 °C.

2.4. Isolation of individual enantiomers of 2-methoxphenidine (2a)

11 × 50 µL injections of racemic 2-methoxphenidine (**2a**, 1 mg/mL in ACN) were resolved using the 150 × 4.6 mm, 5 µm Lux Cellulose-2 column. The combined fractions of the individual enantiomers were collected and evaporated to dryness under vacuum and reconstituted in 500 µL of ACN prior to analysis.

2.5. Software

Log *D* and pK_a values were predicted using ACD/Percepta (Toronto, Canada, version 2019.1.3). Resolution value (R_s) and tailing factor (t_f) was calculated as defined in the United States Pharmacopoeia:

$R_s = 1.18(t_r^2 - t_r^1)/(w_1 + w_2)$

where t_r^1 and t_r^2 are the retention times in minutes of, respectively, the first and the last eluting peak of a pair, while w_1 and w_2 are the widths at half height in minutes of these peaks.

$t_f = w_{0.05}/(2d)$

$w_{0.05}$ = width of the peak at 5 % of the peak height

d = distance between the perpendicular dropped from the peak maximum and the leading edge of the peak at 5 % of the peak height

3. Results and discussion

Chromatographic and electrophoretic chiral method development strategies typically employ generic protocols in order to minimize time-consuming trial-and-error approaches, [24,27]. A commonly employed chiral HPLC strategy using polar organic solvent chromatography (POSC) conditions consists of the rapid screening of analytes on a range of differing polysaccharide based chiral stationary phases (CSPs) with a limited number of POSC mobile phases. The latter normally contain both basic and acidic mobile phase additives such as 0.1 % v/v DEA and FA as they have been demonstrated to enhance the enantiomeric separation selectivity and improve peak shape and reduce column memory effects [16,24,27]. However, the effect of these simple additives on enantiomeric separations is more complex than first thought [26,28]. The results from these generic screening strategies can then form the basis of an excellent starting point for further optimization of the parameters controlling enantiomeric resolution. For example, temperature, the type, concentration, and ratio of the acidic:basic additive as well as the type of organic modifier in the mobile phase are important parameters that can be fine-tuned to optimize retention and enantiomeric resolution [29].

The differing polysaccharide-based CSPs facilitate enantiomeric separation as a result of the analyte enantiomers engaging in differing interactions within the chiral cavities of the polymeric polysaccharide derivative [30]. For example, steric, dipole–dipole, π–π and hydrogen bonding interactions, of differing strength, which occur within the polysaccharide cavities are thought to be responsible for enantiomeric resolution. The concept of the cavity is widespread in chiral chromatography with a polysaccharide-based chiral selector (CS). However, its role in chiral discrimination is not so much size exclusion but rather optimal or sub-optimal spacing of the various structural moieties of the CS that can interact with a complementary functionality on the chiral molecule. Such optimal spacing for one of the enantiomers results in preferential retention and ultimately resolution. Hence, the term cavity is essentially equating to the adequate topology of functional groups capable of interacting with the chiral analyte within the layer of the CS. This is not in conflict with the concept of steric hinderance since the latter term may be interpreted as inadequate topology. As a result of the plethora of interactions that an analyte may undergo with a CSP, predicting or explaining chiral discrimination is extremely complex, if not, impossible; this being the reason why generic chiral screening approaches are so popular. Differing polysaccharide-based CSPs have been demonstrated to exhibit contrasting enantiomeric selectivity due to the nature and position of their specific substituents on their phenyl ring [16].

There have been limited studies on the direct enantiomeric separation of only a few diphenidines. Capillary electrophoresis using cyclodextrins as CS has been reported to separate the enantiomers of (**1**) and (**2a**) [21]. Chiral HPLC using a Lux Cellulose-2 CSP consisting of cellulose *tris*(3-chloro-4-methylphenylcarbamate) coated on silica gel has been successfully used in the POSC mode [15,16] for the enantiomeric separation of (**1**) and (**2a**). In comparison, polysaccharide-based CSPs used in a NP mode were highlighted not to resolve the enantiomers of (**1**) or (**2a**) [18,19]. Recently, the enantiomeric separation of (**2a**) by supercritical fluid chromatography using a vancomycin based CSP [31] or polysaccharide based CSPs [15] has been reported.

Hence, the chiral strategy that was adopted in this study to investigate the enantiomeric separation of 32 novel and structurally different diphenidines (**1–14**, Fig. 1) consisted of evaluating six differing polysaccharide CSPs (similar to the Lux Cellulose-2 CSP that has been previously reported to enantiomerically separate (**1**) and (**2a**)) using a single POSC mobile phase composition of 95:5:0.1:0.1 v/v/v/v ACN/IPA/FA/DEA.

3.1. Effect of temperature on the separation of 2-, 3- and 4-methoxphenidines (2a-2c) and their enantiomers on cellulose tris(3-chloro-4-methylphenylcarbamate) chiral selector {Lux Cellulose-2}

The groups of Taschwer [16] and Jurásek [15] have previously reported the successful enantiomeric separation of (**1**) and (**2a**) respectively using a cellulose based CSP containing cellulose *tris* (3-chloro-4-methylphenylcarbamate) as CS [i.e., Lux Cellulose-2] in the POSC mode with mobile phase composed of 95:5:0.1:0.1 v/v/v/v ACN/IPA/FA/DEA. The separation was performed at ambient and 27 °C respectively but there was no report of the effect of temperature on the enantiomeric separation selectivity [15,16]. Temperature is known to be a critical parameter in controlling enantiomeric selectivity in HPLC [26,32]. For example, the effect of temperature on the kinetic aspects of enantioselectivity is well understood (i.e., efficiency, pressure / viscosity and analyte diffusivity). However, retention and selectivity (i.e., thermodynamic aspects) are not so predictable, for example, the resolution of certain enantiomeric separations increases as temperature decreases while for others the opposite or no effect is observed [26].

The effect of temperature within the range of 15 and 30 °C on the enantiomeric separation methoxy-substituted diphenidines (**2a-2c**) was assessed using the generic POSC chiral HPLC conditions as described for the 2-methoxy regioisomer [15,16]. All the enantiomers of the derivatives (**2a–2c**) were demonstrated to exhibit linear van't Hoff relationships (i.e., the enantiomeric selectivity increased with decreasing temperature, indicating that the CSP-enantiomer recognition was an enthalpy driven process, Supplementary Material 2). However, conclusions drawn based on van't Hoff plots can be deceptive in chiral chromatography since several different types of adsorption sites may be present on the surface of CSP [33].

The latter eluting enantiomers of the regioisomeric methoxphenidines exhibited a greater sensitivity to temperature compared to their earlier eluting enantiomers (see Fig. 2). The 2-methoxphenidine enantiomers eluted with a resolution value (R_s) of 10.5 at 20 °C which corresponds well to that obtained previously at ambient temperature [16] (R_s = 11.5) and at 27 °C [15] (R_s = 9.3) when corrected for equivalent column length. No change in enantiomeric elution order was observed within the temperature range studied. However, peaks **2b²** and **2a** changed elution order as a function of the oven temperature (i.e., enantiomer **2b²** eluted before enantiomer **2a** at 30 °C whereas at temperatures below 20 °C **2a** eluted before **2b²**).

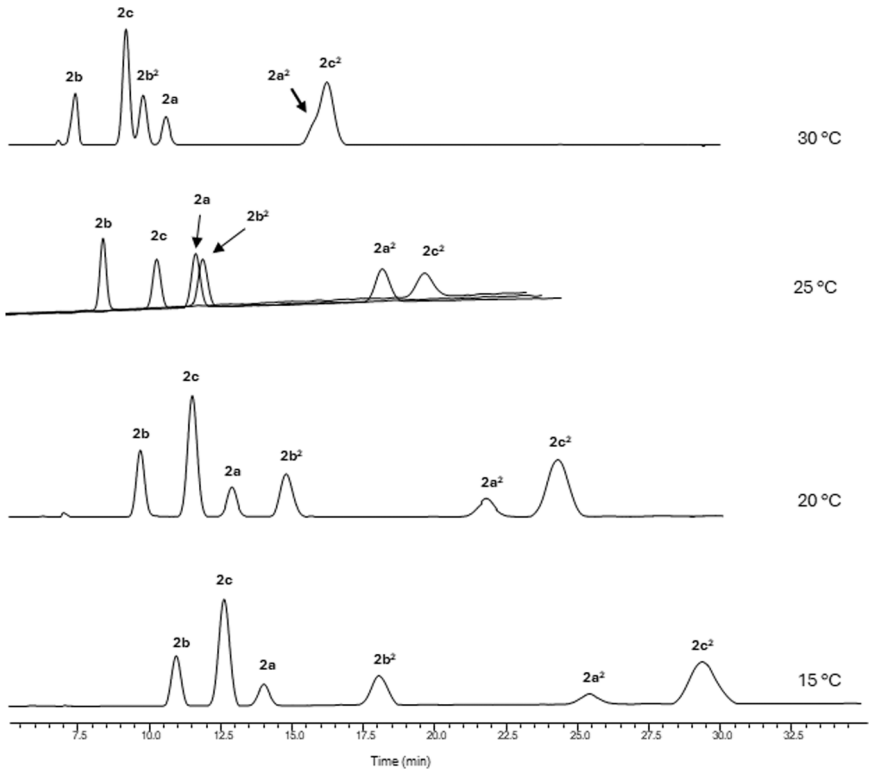

Fig. 2. The effect of column temperature on the chemo and enantiomeric separation of the regioisomeric methoxphenidines (**2a-2c**). Superscript 2 refers to the second peak of the enantiomeric pair. HPLC conditions and sample preparation as described in Sections 2.3 and 2.2.1 respectively using Lux Cellulose-2 CSP.

Temperatures of 25 °C and above failed to separate of all six enantiomers of diphenidines (**2a**–**2c**) in one run, however, at a temperature of 20 °C, excellent separations were achieved for all six enantiomers of the methoxy regioisomers (see Fig. 2, $R_{s\ (min)}$ for the second eluting peaks of **2a** and **2c** (**2a²**, **2c²**) = 1.9) with excellent peak shape (Fig. 2 and Supplementary Material 3) within a reasonable run time.

Table 2
Resolution (R_s) of the monosubstituted diphenidines (**1-10**) as a function of CSP. HPLC conditions as described in Section 2.3 with a column oven temperature of 20 °C.

Compound number	Substituent	Lux Cellulose 1 R_s	Lux Cellulose 2 R_s	Lux Cellulose 3 R_s	Lux *i*-Cellulose 5 R_s	Lux *i*-Amylose 1 R_s	Lux *i*-Amylose 3 R_s
2a	2-Methoxy	0.0	10.5	0.0	0.9	2.8	5.4
2b	3-Methoxy	0.0	8.6	0.0	0.4	1.0	3.6
2c	4-Methoxy	0.9	13.1	0.0	2.4	0.0	3.2
3a	2-Trifluoromethoxy	0.0	1.9	0.0	0.0	0.9	3.0
3b	3-Trifluoromethoxy	1.2	2.9	0.0	0.0	1.2	1.4
3c	4-Trifluoromethoxy	0.0	5.9	0.0	0.9	0.6	2.8
4a	2-Fluoro	0.6	11.9	0.0	0.9	0.6	3.7
4b	3-Fluoro	0.6	8.7	0.0	0.0	2.3	3.3
4c	4-Fluoro	0.0	8.6	0.0	1.0	0.5	3.6
5a	2-Chloro	0.0	5.9	0.0	0.9	2.4	5.1
5b	3-Chloro	0.9	7.7	0.0	0.0	0.0	3.8
5c	4-Chloro	0.0	9.0	0.0	0.7	1.7	4.7
6a	2-Bromo	0.6	4.8	0.0	1.7	1.9	5.2
6b	3-Bromo	0.8	6.3	0.0	0.0	1.0	3.8
6c	4-Bromo	0.0	7.9	0.0	0.5	2.6	3.8
7a	2-Iodo	1.8	2.3	0.0	2.3	1.9	4.5
7b	3-Iodo	0.4	5.6	0.0	0.0	1.0	3.6
7c	4-Iodo	0.0	7.2	0.0	0.8	2.9	4.7
8a	2-Methyl	1.8	7.8	0.0	0.2	2.0	6.3
8b	3-Methyl	0.0	10.9	0.0	1.5	0.5	5.0
8c	4-Methyl	1.2	15.3	0.0	2.3	1.1	3.4
9a	2-Trifluoromethyl	2.5	1.5	0.0	2.4	0.0	1.4
9b	3-Trifluoromethyl	1.1	1.2	0.0	0.0	1.5	2.7
9c	4-Trifluoromethyl	0.0	3.6	0.0	0.3	1.4	2.1
10	Naphthyl	0.0	8.2	0.0	0.8	2.0	4.8
1	Unsubstituted	0.4	22.4	0.0	1.9	1.5	3.3
	Maximum R_s	2.5	22.4	0.0	2.4	2.9	6.3
	Minimum R_s	0.0	1.2	0.0	0.0	0.0	1.4

3.2. Effect of differing chiral selectors and differing diphenidine derivatives on enantiomeric chiral discrimination

It is well known that different polysaccharide-based CSs may exhibit opposing enantiomeric selectivity due to the nature of their differing substituents, their position on the phenyl ring, and type of polysaccharide. Therefore, an evaluation was performed on six differing commercially available polysaccharide CSPs based on either cellulose or amylose, including both coated or immobilized CSs, with ligands of differing functionality (Table 1) for the enantiomeric separation of 26 diphenidine derivatives (**1–10**), possessing either electron donating / withdrawing substituents of varying size and lipophilicity on the 1-phenyl ring (Fig. 1). These included the 2-, 3- and 4-positional isomers (commonly referred to as regioisomers) of eight diphenidine families. It is anticipated that similar separations might be afforded on equivalent CSP from other vendors (e.g. Daicel Chiral Technologies).

The previously reported coated cellulose *tris*(3-chloro-4-methylphenylcarbamate) CS (i.e., Lux Cellulose-2) was observed to generate excellent separation (R_s = 1.5–22.4, Table 2 and Figs. 3 & 4) for 25 of the 26 diphenidines (**1–10**) investigated and partial separation for the 3-trifluoromethyl analogue (**9b**, R_s = 1.2). This CSP provided optimum enantiomeric separation (R_s = 2.9–22.4) for 21 of 26 the diphenidine derivatives evaluated (Table 2 and Fig. 3).

Lux Cellulose-1 and Lux Cellulose-3 CPSs exhibited low retention and hence poor enantiomeric selectivity compared to the other four CSPs (Supplementary Material 1 and 4). Lux Cellulose-3 (a coated CSP based on cellulose *tris*(4-methylbenzoate)), which was the only non-carbamate polysaccharide derivative used as CS in this study, failed to afford any chiral discrimination for any of the monosubstituted diphenidines investigated under POSC conditions (Table 2 and Fig. 3). In contrast, Lux Cellulose-2 and Lux *i*-Amylose-3, Lux Cellulose-5 and Lux *i*-Amylose-1 all exhibited moderate to good retention and enantiomeric selectivity (Supplementary Materials 1,3–7).

Peak shape was observed to be good for all of the 26 diphenidine derivatives, for example, the mean tailing factor (t_f) observed with Lux Cellulose-2 equated to 0.96 and 1.03 for the first and second eluting enantiomers respectively (Supplementary Materials 1,3–7).

Chlorine substituents on the phenyl ring of the CSP have been reported to promote enhanced enantiomer selectivity when employed in the POSC mode [27]. Similarly, the results obtained with the diphenidines highlighted that the 3-chloro-4(5)-methylphenyl moieties attached to cellulose or amylose (i.e., Lux Cellulose-2 and Lux *i*-Amylose-3) were highly important for chiral recognition as replacement of the 3-chloro substituent with a methyl moiety greatly decreased its enantiomeric discrimination (i.e., Lux *i*-Amylose-1) (see Table 2 and Fig. 3). Interestingly, the substitution of two chlorine atoms on the phenyl ring of the polysaccharide derivative (i.e., Lux *i*-Cellulose-5) generated less chiral discrimination than with the 3-chloro-4(5)-methylphenyl substituent patterns.

The enantiomers of all 26 diphenidines could be successfully resolved with a $R_s > 2.5$ on Lux Cellulose-2 (**1, 2a-2c, 3b** and **3c, 4a-4c, 5a-5c, 6b, 6c, 7b, 7c, 8a-8c, 9a, 9c**, and **10**), Lux *i*-Amylose-3 (**3a**, 2-trifluoromethoxy; **6a**, 2-bromo; **7a**, 2-iodo; **9b**, 3-trifluoromethyl) and Lux Cellulose-1 (**9a**, 2-trifluoromethyl) using the standard generic HPLC conditions proposed here, at 20 °C (Fig. 4).

Diphenidine derivatives possessing electron donating substituents [i.e., methoxy- (**2a-2c**), methyl- (**8a-8c**) and naphthyl- (**10**)] yielded retention similar to the unsubstituted derivative (**1**). In contrast, the enantiomers of the diphenidines with electron withdrawing substituents [i.e., trifluoromethoxy- (**3a-3c**), halogenated (**4a-4c**, **5a-5c**, **6a–6c** and **7a-7c**) and trifluoromethyl- (**9a-9c**)] eluted significantly earlier (Supplementary Materials 1,3–7) highlighting a reduced interaction with the CS. In many cases, this led to a corresponding decrease in enantiomeric selectivity, however, this was not always the situation (see Table 2).

In general, substitution in the 2-position of the 1-phenyl ring (i.e., *ortho*-substituted 1, 2-diarylethylamines) resulted in lower enantiomeric selectivity, which may be attributed to reduced inclusion into the polysaccharide cavities as a result of steric restrictions. In contrast, substitution in the 4-position of the 1-phenyl ring (i.e., *para*-substituted 1,2-diarylethylamines), resulted in enhanced retention and enantiomeric separation, presumably due to stronger interaction and / or inclusion into the cavities of the polysaccharide. Alternatively, it may be due to non-chiral interactions such as π−π interactions supporting the formation of a diastereomeric complex [18]. Similar effects have been reported for other regioisomeric NPS such as 4-chloromethcathinone or 4-methylcathinone, compared to their corresponding 2-substituted analogues [16]. The 3-substituted diphenidine derivatives gave results in between the 2 and 4-substituted analogues (see Fig. 4).

The retention of the 3- and 4-substiuted halogenated diphenidines (**4–7**) on the CSPs, which demonstrated enantiomeric selectivity (i.e., Lux Cellulose-2, Amylose-3, Cellulose-5 and Cellulose-1), was observed to increase as the atomic size of the substituted halogen increased. However, for the 2-iodo analogue (**7a**), this resulted in a decrease in retention compared to the 2-bromo analogue (**6a**) presumably due to a steric effect on inclusion into the polysaccharide cavities

Fig. 3. Chiral performance of the CSP against the 26 chiral monosubstituted diphenidine racemates (**1–10**). Compounds separated with a resolution > 1.5. Frequency that the CSP provided optimum resolution. Compounds that failed to be resolved (i.e., resolution = 0).

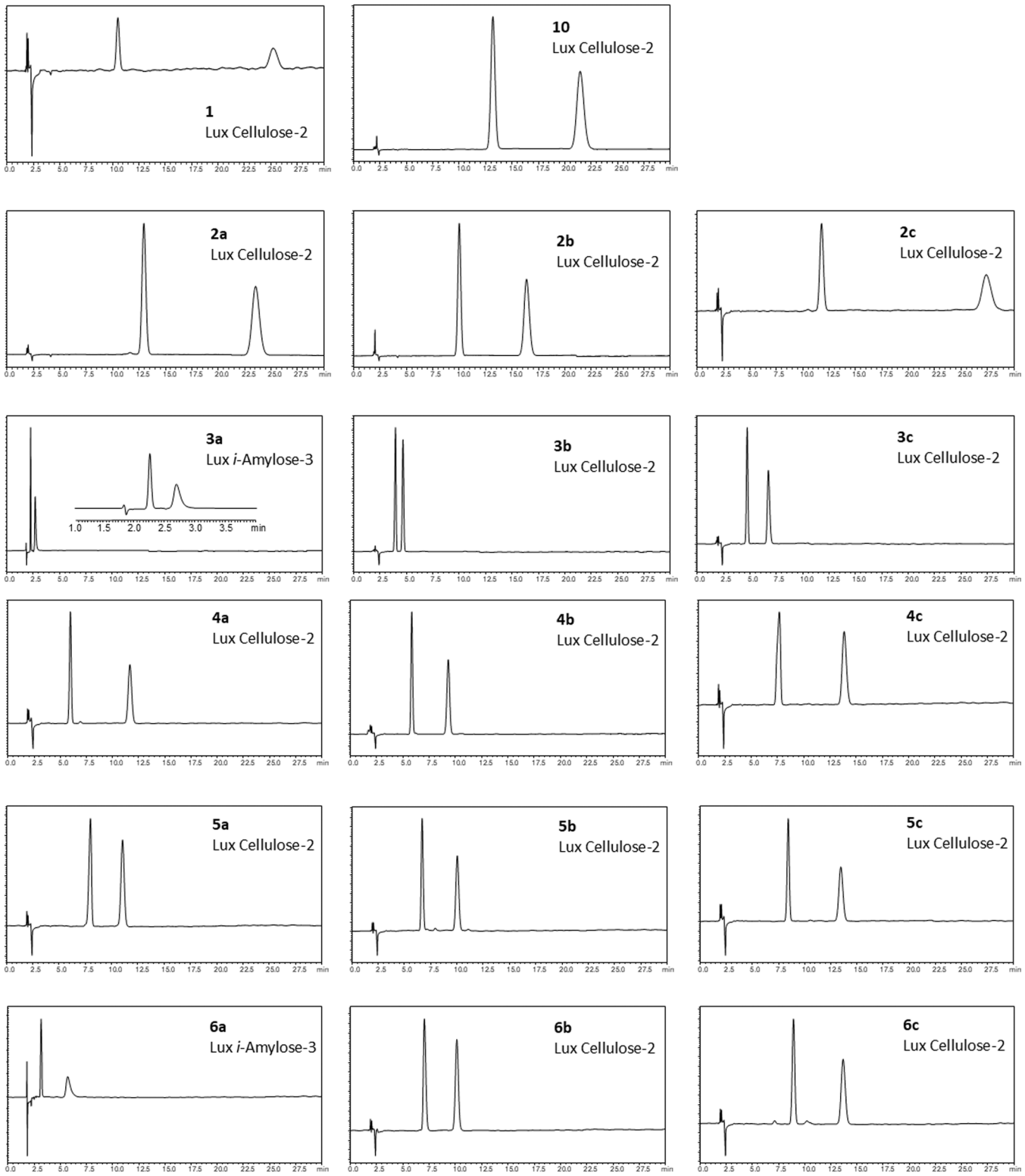

Fig. 4. Enantiomeric separation of the monosubstituted diphenidines (**1–10**) as a function the CSP. HPLC conditions and sample preparation as described in Sections 2.3 and 2.2.1 respectively with a column oven temperature of 20 °C. Optimum CSP specified in each chromatogram.

(Supplementary Materials 1,3–7).

3.3. 2-Methoxphenidine (2a) enantiomer elution order as a function of the chiral stationary phase

Reversal of the enantiomer elution order (EEO) has been demonstrated with differing CSs [28]. However, enriched samples of the

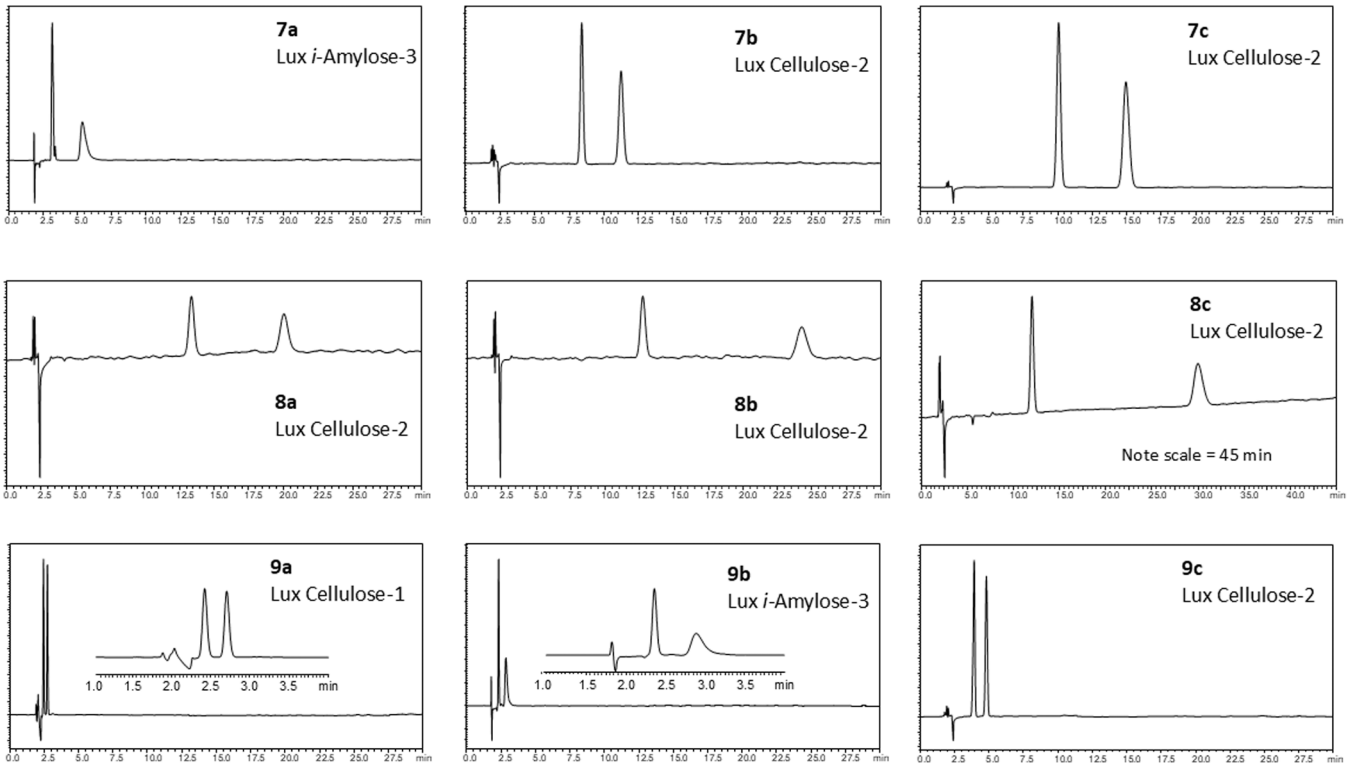

Fig. 4. (*continued*).

2-methoxphenidine **(2a)** enantiomers were required to investigate this. These enantiomers (**2a**) have reportedly been resolved by fractional recrystallization using (-)-2,3-dibenzoyl-L-tartaric acid monohydrate [15], however, this was not successfully reproduced. Instead, small amounts of individual enantiomers were isolated by multiple HPLC overload experiments on the analytical Lux Cellulose-2 column. This provided enantiomers with enantiomeric excesses > 99 % for the first and second peaks. Reference [15] demonstrated that the (R)- and (S)-configurations of **2a** could be assigned to the first and second eluting enantiomers using HPLC with Lux Cellulose-2 CSP @ 27 °C.

The individual (R)- and (S)-enantiomers of 2-methoxphenidine (**2a**) were demonstrated to exhibit the same elution order when chromatographed on cellulose-based CSP (i.e., Lux *i*-Cellulose-5 and Lux Cellulose-2), whereas a reversal of the EEO was observed on amylose-based CSPs (i.e., Lux *i*-Amylose-1 and Lux *i*-Amylose-3). This highlighted the importance of the polysaccharide cavity in providing the optimal or sub-optimal spacing of the various structural moieties of the CS that can interact preferentially with a complementary functionality on one of the enantiomers. The interaction of the (S)-enantiomer of (**2a**) is stronger with the cellulose-based CSP whereas, with the amylose-based CSP, the reverse is observed (Supplementary Material 8 for exemplar chromatograms).

Interestingly, Lux Cellulose-1 (based on cellulose *tris*(3,5-dimethylphenylcarbamate as CS) exhibited no chiral discrimination of the enantiomers of (**2a**) whereas Lux *i*-Amylose-1, which is based on a similar derivative, but of amylose (namely, amylose *tris*(3,5-dimethyphenylcarbamate)), displayed good separation. These diverging results highlight the important role played by the polysaccharide for chiral discrimination. With both chiral selectors, the functional groups available for selector / selectant interactions are the same, nevertheless, their spatial arrangement, specifically their spatial availability for interacting, must be drastically different because of the specific configuration adopted by each polysaccharide derivative. These findings suggest that conclusions regarding the (R)- or (S)-configuration of the diphenidine enantiomers based on their elution order alone may be erroneous.

3.4. Forensic application of the chiral HPLC method to seized street samples of 2-methoxphenidine (2a) and diphenidine (1)

Eight seized samples were provided to MANDRAKE, between June – October 2016 and October – December 2023, by Greater Manchester Police and were analysed using a previously described method [5] to confirm both their authenticity and purity. GC-MS analysis confirmed that four samples contained diphenidine (R_t = 23.7 min, *m/z* (base peak) = 174 $[M + H]^+$) and four contained 2-methoxphenidine (R_t = 28.1 min, *m/z* (base peak) = 204 $[M + H]^+$) respectively, with no apparent adulteration (> 98.7 % purity, by GC-MS, in all cases). The described chiral HPLC-methodology was used to unambiguously confirm the racemic nature of these eight seized street samples purported to contain either diphenidine (**1**) or 2-methoxphenidine (**2a**) (Fig. 5 for representative chromatograms – the four 2-methoxphenidine and four diphenidine samples generated identical chromatograms to the standard compounds **2a** and **1** respectively). Commonly used adulterants found in street samples [34], such as benzocaine (R_t = 2.0 min), paracetamol (R_t = 2.6 min), caffeine (R_t = 6.3 min) and procaine (R_t = 7.5 min) were demonstrated not to interfere with the enantiomeric analysis of 2-methoxphenidine (**2a**) and diphenidine (**1**) on Lux Cellulose-2 CSP.

3.5. Chiral separation of di- and trisubstituted diphenidines on the cellulose tris(3-chloro-4-methylphenylcarbamate) {Lux Cellulose-2}

The generic methodology using Lux Cellulose-2 CSP was then further applied to the chiral analysis of four di- (**11a**, **11b**, **12a** and **12b**) and two trisubstituted diphenidines (**13** and **14**). Chiral resolution of the structural isomers of the two methylenedioxy-substituted 1,2-diarylethylamines (**11a** and **11b**) was achieved using the generic conditions at 20 °C (Fig. 6). The 2, 3- and 3, 4-methylenedioxy derivatives (**11a** and **11b** respectively) yielded impressive R_s values of 8.9 and 10.2 respectively with excellent peak shape (t_f ranging from 1.0–1.2).

As observed previously with the monosubstituted diphenidines, the

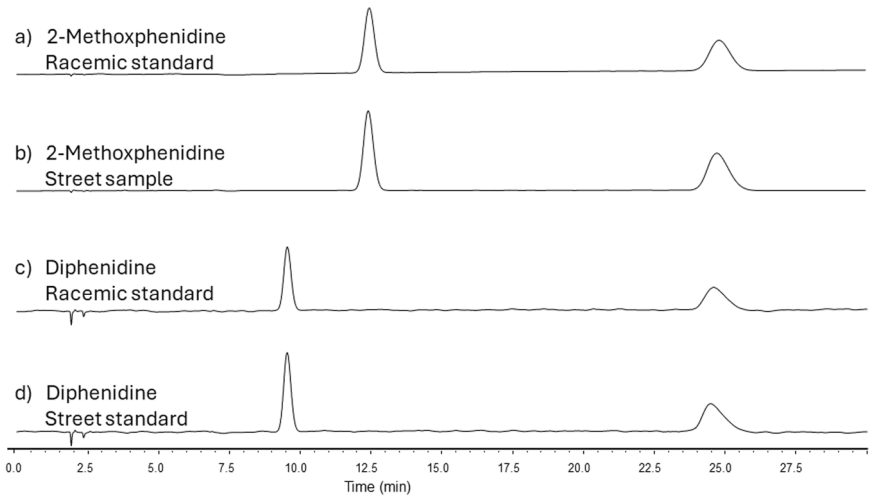

Fig. 5. Enantiomeric separation of a typical diphenidine and 2-methoxphenidine street samples (**1** and **2a**) and of racemic standards on Lux Cellulose-2 CSP at 20 °C. HPLC conditions and sample preparation as described in Sections 2.3 and 2.2.1 respectively.

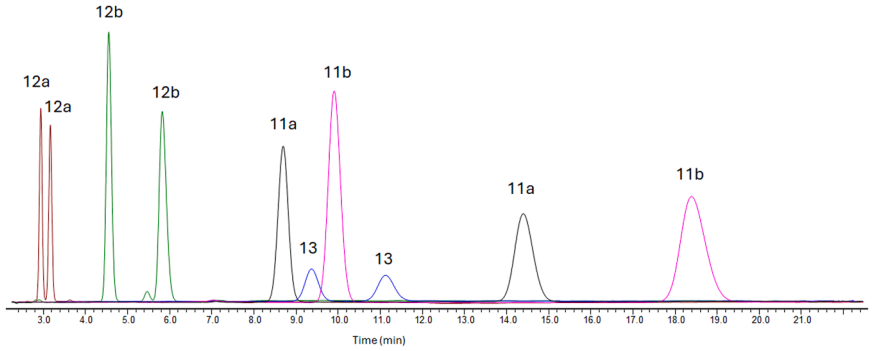

Fig. 6. Enantiomeric and chemo separation of di- and trisubstituted diphenidines (**11a** and **11b, 12a** and **12b** and **13**) on Lux Cellulose-2 CSP at 20 °C. HPLC conditions and sample preparation as described in Sections 2.3 and 2.2.1 respectively. UV detection wavelength = 296 nm.

corresponding difluoro-analogues (**12a** and **12b**) exhibited reduced retention along with reduced chiral discrimination (Fig. 6 and Supplementary Material 9). The 2,3- and 3,4-difluorinated analogues (**12a** and **12b**) yielded R_s values of 1.7 and 4.7 respectively with excellent peak shape (t_f ranging from 1.0–1.2).

The trimethoxy analogue (**13**) afforded excellent chiral separation as shown in Fig. 6 with a R_s value of 2.6, however, in comparison, the 2,5-dimethoxy-4-methyl-trisubstituted analogue (**14**) failed to afford enantiomeric separation on Lux Cellulose-2 despite its good retention (R_t 13.7 min). Co-elution was confirmed by the fact that only one peak was obtained even when an extended run time of 120 min was employed. The peak area was comparable to that of the combined peak areas for the two enantiomeric peaks when chiral discrimination was obtained (Supplementary Material 10). A good resolution value was achieved for trisubstituted diphenidine (**14**) on Lux *i*-Amylose-3 CSP (Fig. 7, $R_s = 3.0$) and only partial separation on Lux *i*-Amylose-1 ($R_s = 1.2$), Lux Cellulose-1 ($R_s = 1.0$), Lux *i*-Cellulose-5 ($R_s = 0.8$), whereas co-elution was observed on Lux Cellulose-3 (Supplementary Material 10).

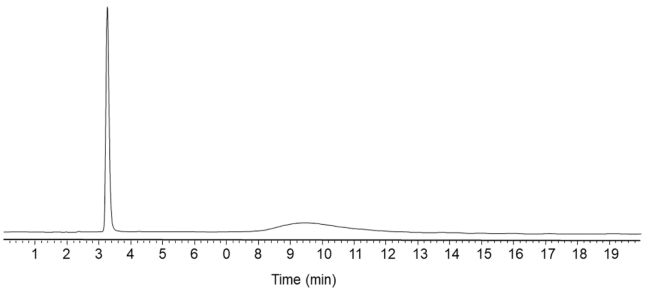

Fig. 7. Enantiomeric separation of the trisubstituted diphenidine (**14,** $R_s = 3.0$) on Lux *i*-Amylose-3 CSP at 20 °C. HPLC conditions and sample preparation as described in Sections 2.3 and 2.2.1 respectively using Lux Cellulose-2 CSP, UV detection wavelength = 296 nm.

3.6. Chemo- and enantiomeric selectivity

Chiral columns, in addition to providing enantiomeric selectivity (i.e., separation of enantiomers), can exhibit chemoselectivity (i.e., separation of the regioisomers) depending on the type of CSP employed [35]. The described chiral HPLC methodology provided separation of all six components (two enantiomers for each of the three regioisomers) for five out of the eight monosubstituted diphenidine regioisomers [methoxy- (**2a-2c**), chloro- (**5a-5c**), bromo- (**6a-6c**), iodo- (**7a-7c**) and methyl- (**8a-8c**)]. For the remaining three regioisomers [trifluoromethoxy- (**3a-3c**), fluoro- (**4a-4c**) and trifluoromethyl- (**9a-9c**)] partial separation or co-elution of components was observed. However, if required, the latter regioisomers can be easily separated by either HPLC [6] or supercritical fluid chromatography [13] for confirmatory purposes. The combination of the described achiral and chiral methodologies into a single 2D-LC RP/POSC separation may be an attractive proposition. Lux Cellulose-2 CSP demonstrated chemo and enantiomeric selectivity for the disubstituted diphenidine structural isomers (i.e., **11a, 11b, 12a** and **12b** Fig. 6).

The type of chiral columns investigated have a pressure rating of 310 bar, hence, if required, resolution could be improved by a factor of 1.7 by simply employing a 3 μm x 25 cm column since the current column format (5 μm x 15 cm) only exhibited a back pressure < 60 bar.

4. Conclusion

The direct enantiomeric HPLC separation of 32 diphenidines on a range of six polysaccharide-based stationary phases under POSC conditions was evaluated. A single mobile phase combined with Lux Cellulose-2 and Lux *i*-Amylose-3 columns afforded baseline separation of all 32 diphenidines. Lux Cellulose-2 CSP was the most successful CSP in that it afforded baseline and optimum separation of 25 and 21 of the 26 monosubstituted diphenidines respectively. Temperature was demonstrated to be an important parameter in controlling enantiomeric chromatographic selectivity. Enantiomeric elution order reversal was observed with 2-methoxphenidine (**2a**), the (R)- and (S)-configurations eluted in the order (R)- before (S)- with cellulose-based CSPs, whereas the reverse occurred with the amylose-based CSPs. Enantioselectivity and chemoselectivity of mono and disubstituted diphenidine regioisomers was highlighted. Street samples of 2-methoxphenidine and diphenidne were confirmed as being traded in their racemic form. Commonly used adulterants in powered samples were shown not to interfere in the analysis.

CRediT authorship contribution statement

Andrew Costello: Resources. **Benjamin S. Barrett:** Writing – review & editing, Investigation, Formal analysis. **Michael. A. Sallenger:** Formal analysis. **Tivadar Farkas:** Writing – review & editing, Resources, Conceptualization. **Oliver B. Sutcliffe:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Conceptualization. **Melvin R. Euerby:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Investigation, Formal analysis, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2025.116728.

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