Guilty by Dissociation: Part B: Evaluation of Supercritical Fluid Chromatography (SFC-UV) for the analysis of regioisomeric diphenidine-derived Novel Psychoactive Substances (NPS).

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Highlights

- Rapid analysis (<10 mins) of regioisomeric diphenidines by gradient SFC
- Six of the eight substituted regioisomeric groups were separated by SFC
- Elution order of regioisomers is independent of stationary phase chemistry
- Elution order of the regioisomers is dependent on nature of substituent
- SFC generated orthogonal elution of the diphenidines compared to RP-UHPLC

Abstract

Supercritical Fluid Chromatography (SFC-UV) employing a carbon dioxide (CO_2) and 10 mM ammonium acetate (pH unadjusted) in MeOH-water (95:5 v/v) gradient provides a rapid analysis (t_G <10 mins) of 31 novel, regioisomeric diphenidine-derived psychoactive substances, on a range of stationary phases of differing polarity. Medium to large selectivity differences between regioisomers, was observed on the acidic, neutral and basic SFC phases. The greatest selectivity differences were obtained with the acidic and basic phases and the smallest between acidic and neutral phases. For individual substituted *ortho*-, *meta*- and *para*-isomers, the same elution order was observed irrespective of the nature of the stationary phase. This suggested that partitioning into the aqueous layer surrounding the stationary phases as well as the electrostatic interactions between the protonated diphenidine derivatives and the stationary phase, may be the primary retention mechanisms under these SFC conditions. The acidic silica stationary phases yielded longer retention of the analytes *via* electrostatic attraction, whereas the basic phases resulted in shorter retention *via* electrostatic repulsion. SFC effected acceptable separation of six of the eight substituted groups of *ortho*-, *meta*- and *para*-diphenidines evaluated. As the size of the halo-substituent increased, the resolution between *ortho*-/*meta*-isomers decreased, resulting in co-elution of the *ortho*- and *meta*-bromodiphenidines. Fluphenidines and chlorodiphenidines generated an elution order of *meta*- < *ortho*- < *para*- whereas an elution order switch was observed for the iodophenidines. This contrasted with RP-UHPLC where the elution order for the fluphenidines and iodophenidines was *para*- < *ortho*- < *meta*- and *para*- < *meta*- < *ortho*- respectively. An orthogonal elution order of diphenidines was demonstrated between the RP-UHPLC and SFC stationary phases due to the polarity differences between the separation modes. In general, hydrophilic compounds, which were poorly retained on a C18 reverse phase column, were well retained on SFC columns.

Keywords: Forensic; illicit drugs; regioisomers; diphenidines; novel psychoactive substances; SFC

1.0 Introduction

Novel psychoactive substances (NPS) are entities 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 like those posed by the substances covered by the conventions [1]. 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-Diarylethamines, such as 1-(1, 2-diphenylethyl)piperidine (diphenidine, **1**) and 1-[1-(2-methoxyphenyl)-2-phenylethyl]piperidine (2-methoxphenidine, 2-MXP, **2**) (Fig. 1) are dissociative, psychoactive substances, encountered in both tablet or powder forms, which distort perceptions, produce feelings of detachment, and induce a state of anaesthesia by antagonising ionotropic *N*-methyl-D-aspartate receptors (NMDAR) in the central nervous system [2]. Though the supply and production of **1** and **2** are now controlled in the United Kingdom by the Psychoactive Substances Act (2016), the emergence of novel diphenidine derivatives, such as 1-[1-(2-chlorophenyl)-2-phenylethyl]piperidine (2-chlorodiphenidine, 2-Cl-DPH, **17**), still raises considerable legal and analytical challenges in both the forensic identification and regioisomeric discrimination of these materials, due to their inference in several fatalities [3 - 5]. Analytical differentiation of regioisomers is a significant challenge within forensic drug analysis but can be readily achieved using Nuclear Magnetic Resonance (NMR) spectroscopy [6], however, only a small number of laboratories have such instruments. The discrimination of specific regioisomers using this technique is both cost and labour intensive when compared to the gas chromatography-mass spectrometry (GC) [7], reversed-phase high performance liquid chromatography (RP-HPLC) [8, 9] and ultra-high performance liquid chromatography-mass spectrometry-mass spectrometry (UHPLC-MS/MS) [10] approaches which have been applied to a number of regioisomeric 1, 2-diarylethylamines.

Recently, the technique of Supercritical Fluid Chromatography (SFC) which is a *"hybrid chromatographic technique between LC and GC"* that potentially possesses the advantages of both techniques, has been used in the rapid analysis of certain psychoactive substances (including amphetamine and cathinone derivatives) [11 – 13]. SFC uses a supercritical fluid (SF) such as carbon dioxide (CO_2) at temperatures > 31 °C and pressures > 74 bar as the mobile phase. Supercritical CO_2 exhibits ideal chromatographic properties for a mobile phase, as it possesses the density of a liquid, and the low viscosity and high diffusivity of a gas. Due to

supercritical CO_2 being relatively non-polar, MeOH is typically added (up to 40% v/v) to increase the solvating power of the mobile phase to analyse more polar compounds (SFC has been successfully used for analytes possessing LogP values in the range of -1 to 7). These factors result in a separating technology of extremely high resolving power (i.e. long columns packed with small particles can be utilised due to the inherent low pressure drop), rapid analysis (i.e., short columns packed with small particles at high flow rates) [14] and complementary chromatographic selectivity to the ubiquitous reversed-phase liquid chromatography (RPLC) [15, 16]. SFC is very attractive for many industries as it is attractive for many due to the low toxicity of the mobile phase and its low impact on the environment. To date, SFC has been successfully employed in the pharmaceutical [17, 18], environmental [19] and toxicology/forensic [20, 21] sectors.

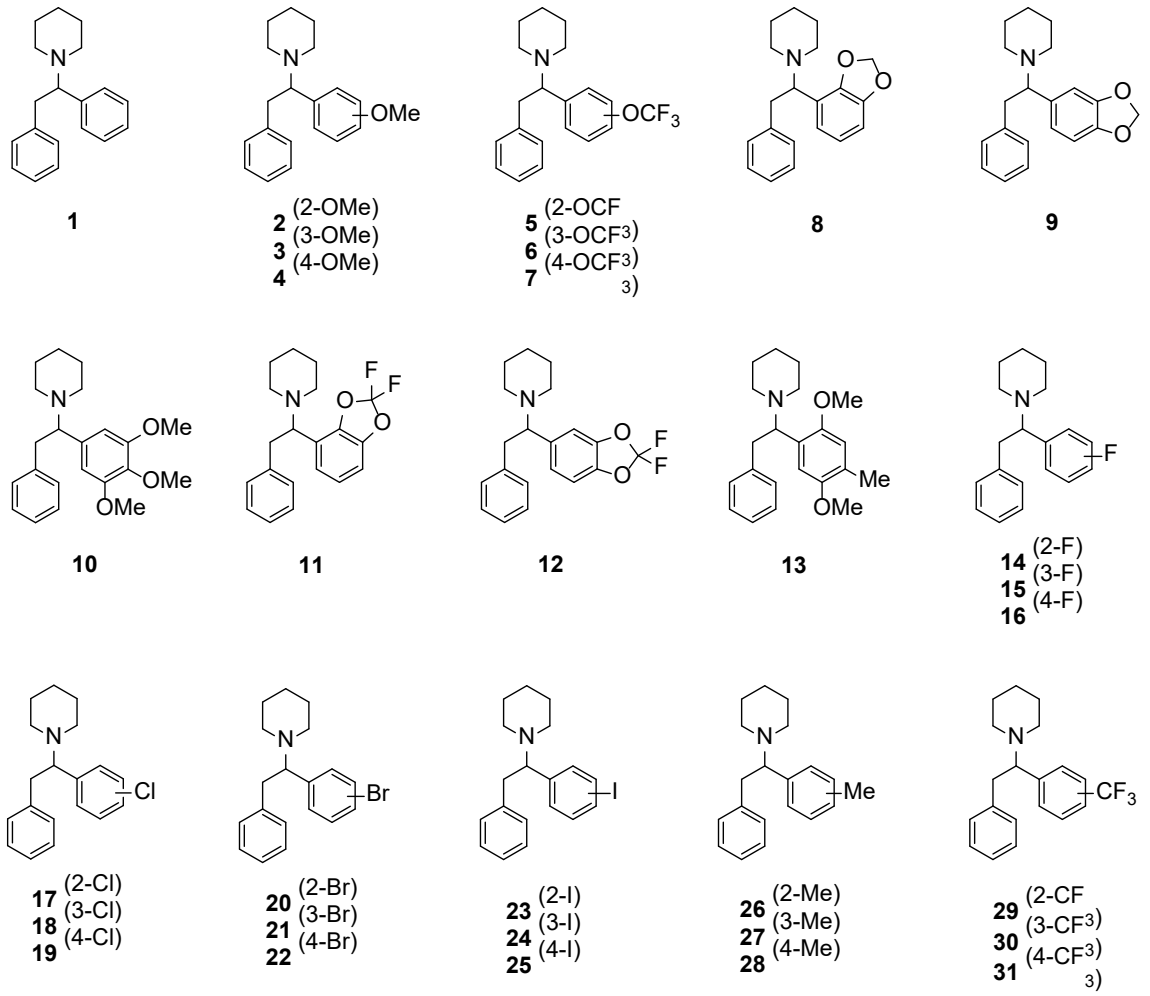

Fig. 1. Structures of the regioisomeric diphenidines (**1** – **31**) utilised in this study.

This paper describes the first reported evaluation of SFC to separate 31 substituted derivatives of widely differing physical and chemical properties using a rapid generic gradient SFC

methodology and compares it to their analysis by liquid chromatography [9, 10]. The library included a range of derivatives possessing either electron donating/withdrawing substituents, commonly included in combinatorial libraries, of varying size and lipophilicity on the phenyl ring (see Fig. 1). The 2-, 3- and 4-positional isomers (commonly known as the *ortho*-, *meta*- and *para*-regioisomers) of eight 1,2-diarylethylamines families (**2 – 4**, **5 – 7**, **14 – 16**, **17 – 19**, **20 – 22**, **23 – 25**, **26 – 28** and **29 - 31**) and two groups of twinned structural isomers (**8**/**9** and **11**/**12**) were synthesised [7, 10] to separate these isomeric compounds and to elucidate if their elution order was dependent on the position and type of substituent on the phenyl ring. The racemic diphenidines were analysed on a range of nine acidic, basic and neutral SFC stationary phases (Table 1) which have previously been demonstrated to exhibit differing chromatographic selectivity [22]. The chromatographic selectivity using SFC conditions was compared to those previously reported using RP-UHPLC [10].

2.0. Materials and Methods

2.1. Synthesis of Standards

All solvents and reagents were of commercial quality (Sigma-Aldrich, Gillingham, UK or Fluorochem Limited, Hadfield, UK) and used without further purification. Diphenidine (**1**) and its derivatives (**2** – **31**, Fig. 1) were prepared as their corresponding hydrochloride salts by MANchester DRug Analysis and Knowledge Exchange (MANDRAKE). The synthesis of the racemic target compounds was achieved using the previously reported method [7, 10] and isolated as their corresponding hydrochloride salts. To ensure the authenticity of the materials utilized within this study, the 31 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 analytical data for compounds **1** – **31** has been previously reported [7, 10].

2.2. Supercritical Fluid Chromatography (SFC)

Supercritical Fluid Chromatography (SFC) was performed on a Shimadzu Nexera-UC (Shimadzu UK Ltd, Milton Keynes, UK) equipped with SFC CO_2 pump (LC-30ADSF), binary pump (LC-30AD) and proportionating values, degassers (DGU-20ASR), autosampler (SIL-30AC), Prominence column oven (CTO-20AC), diode array detector (SPD-M30A) and communication bus module (CBM-20A). The software used to control the SFC system was LabSolutions (Shimadzu UK Ltd, version 5.89). *Sample preparation:* Stock solutions of diphenidine (**1**) and its derivatives (**2** – **31**) were prepared at a concentration of 1 mg mL^{-1} in

MeOH. The individual diphenidine isomers, or mixtures thereof, were diluted to 63 µg mL^{-1} (of each isomer) with MeOH for the chromatographic studies. *Test mixture preparation (for acid/neutral/base suitability tests):* Stock solutions of caffeine, ibuprofen, flurbiprofen, propranolol hydrochloride, atenolol hydrochloride, amitriptyline hydrochloride, naphthalene and bendroflumethiazide were made up in MeOH at a concentration of 1 mg mL^{-1}. A mixture of the stock solutions was prepared and then diluted to 19 - 373 µg mL^{-1} with MeOH for the chromatographic studies.

2.3. Standard Supercritical Fluid Chromatographic conditions

At least 20 column volumes of 2-propanol (IPA) were flushed through the new columns prior to use. 100 column volumes of the appropriate mobile phase were then flushed through the columns prior to commencing the testing, or on changing the mobile phase conditions. The integrity of all the columns was confirmed periodically throughout the experiments by injecting an acid/neutral/base suitability test mixture (see Section 2.2) before and after the experiments. Mobile phases A and B corresponded to carbon dioxide and 10 mM ammonium acetate (pH unadjusted) in MeOH-water (95:5 v/v) respectively. Unless otherwise stated the following SFC conditions were employed: flow rate of 3 mL min^{-1}, temperature of 40 °C, 1 µL injection, back pressure regulation 150 bar. Generic gradient SFC conditions were employed: Initial 1 minute hold at 5% B prior to commencing a linear gradient from 5 - 40% B over 10 minutes, followed by a hold at 40% B for 1 minute, a linear gradient 40 - 5% B over 1 minute and a hold at 5%B for 5 minutes to re-equilibrate the column. The diode array detector was set to monitor a wavelength of 214 nm (bandwidth 8 nm), reference 360 nm (bandwidth: 100 nm). The data sampling rate was set at 40 Hz. Chromatographic values reported are the average of duplicate injections. All columns were new as supplied by the ES Industries (West Berlin, NJ, USA) or Sigma-Aldrich/Merck (Gillingham, UK) (see Table 1) and were 3 µm fully porous materials unless otherwise stated.

2.4. Ultra-High Performance Liquid Chromatography (UHPLC)

UHPLC analysis was performed using an ACE Excel C18 (1.7 µm, 100 Å, 100 x 3 mm i.d. formats) column (Advanced Chromatography Technologies, Aberdeen, UK), employing the chromatographic conditions previously described by Field *et al.* [10], on a Shimadzu Nexera XR UHPLC (Shimadzu UK Ltd) equipped with two binary pumps (LC-30AD) equipped with proportionating valves, degassers (DGU-$20A_{5R}$), autosampler (SIL-30AC), Prominence column oven (CTO-20AC), diode array detector (SPD-M30A) equipped with a 1 µL / 10 mm

pathlength flow cell, 40 µL mixer (dwell volume = 342 µL, system volume = 14 µL [10]) and communication bus module (CBM-20A). The system was controlled, and data collected by means of LabSolutions software (Shimadzu UK Ltd, version 5.86).

2.5. Software

LogD, pK_a and pH values were predicted using ACD/Percepta and ACD/pH calculator software (Toronto, Canada, version 2019.1.3). Principal Component Analysis (PCA) and Hierarchical cluster analysis (HCA) were performed using SIMCA (Sartorius UK Ltd, Surrey, UK, version 14.1).

3.0 Results and Discussion

Reference samples of 31 diphenidine derivatives (**1** – **31**, Fig. 1) were prepared as their corresponding hydrochloride salts using the previously reported synthetic approach from prerequisite aromatic aldehydes as stable, colourless to off-white powders [7, 10]. To ensure the authenticity of the materials utilized within this study, the 31 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.

Table 1. Stationary phases evaluated using the generic SFC conditions (Section 2.3).

Column	Character	Manufacturer	Comments/Functionality	Code
Ascentis Express HILIC	Acidic	Sigma / Merck	Superficially porous silica 2.7 µm	HILIC
GreenSep Silica	Acidic	ES Industries	Silica	Silica
GreenSep Diol	Neutral	ES Industries	Diol	Diol
GreenSep Cyano	Neutral	ES Industries	Cyano	Cyano
GreenSep Ethyl Pyridine	Basic	ES Industries	2-Ethylpyridine	2-EP
GreenSep 4-Ethyl Pyridine	Basic	ES Industries	4-Ethylpyridine	4-EP
GreenSep Pyridyl Amide	Basic	ES Industries	Pyridyl amide	PA
GreenSep DEAP	Basic	ES Industries	Diethylaminopropyl	DEAP
GreenSep Basic	Basic	ES Industries	Imidazole	Basic

Selectivity in SFC can be controlled by changing the mobile phase composition, temperature and pressure [23-25], however, the stationary phase was reported to be the most influential parameter in affecting chromatographic selectivity [26-28]. Hence, a generic gradient SFC condition that is typically used in screening activities was employed [26, 27, 29] to assess the chromatographic retention and separation of **1 – 31** on a range of disparate SFC stationary

phases. Except for the superficially porous Hydrophilic Interaction LIquid Chromatography (HILIC) phase (see Table 1), all stationary phases were fully porous and from the same manufacturer (it was assumed that the same type of base silica had been employed) hence, selectivity differences observed should be directly attributed to the different ligands. Two neutral (i.e. cyano and diol ligands) and five basic phases (i.e. weakly basic 2- and 4-ethylpyridine, pyridyl amide and the strongly basic imidazole and diethylamino ligands) were chosen in addition to two acidic silica phases (see Table 1) [22].

Generic gradient SFC conditions which have previously been reported to generate good peak shapes were employed, these utilised CO_2 and 10 mM ammonium acetate (pH unadjusted) in MeOH-water (95:5 v/v) respectively [27, 29]. A small amount of water was included as this has been shown to yield more reproducible retention times and enhanced peak shapes [29-32]. Supercritical carbon dioxide-MeOH mixtures have been shown to have an approximate pH of 5 (based on colour changes of ionisable dyes) due to the presence of methoxylcarbonic acid [33]. The pH of the supercritical CO_2-MeOH mobile phase during gradient chromatography becomes more acidic as the proportion of MeOH increases. The presence of ammonium acetate in the CO_2-MeOH mixtures does not change the apparent pH, but provides buffering capacity generating more reproducible results [29]. It is believed that in addition to MeOH and CO_2, water and ammonium acetate are adsorbed onto the surface of polar stationary phases in a similar manner to that observed in HILIC providing a pseudo-stationary phase. The presence of 10 mM ammonium acetate increases the thickness of this aqueous layer providing more capacity for the analytes to partition into the water layer. In addition, adsorbed ammonium ions may shield the ionised silanol groups on the silica surface from the protonated basic analytes or the acetate ions may form ion pairs with the protonated basic analytes minimising secondary electrostatic interactions and generating improved peak shape.

A range of eight analytes (i.e., two acidic, three neutral and three basic [two secondary and one tertiary base]) were selected to evaluate the chromatographic selectivity differences of the nine SFC stationary phases. Analyte LogD values ranged from -2.84 to 3.37 at pH 5. A Principal Component Analysis (PCA) score plot (see Supplementary Information Fig. S1) of these analytes' LogD values at pH 5 (assumed pH of SFC mobile phase conditions), in addition to eight other physical and/or chemical parameters, confirmed their disparate nature which was deemed necessary to assess the differences in chromatographic selectivity between the stationary phases. Flurbiprofen and ibuprofen were shown to be similar in their properties but

were included in the test mixture to assess the chromatographic discriminating power of the differing phases to analytes of similar properties.

3.1 SFC retention and selectivity evaluation of the stationary phases using an acid/neutral/base suitability test mixture

The hydrophobic, neutral analyte naphthalene (LogD = 3.37 at pH 5) failed to be retained on any of the polar SFC columns and was deemed a suitable void volume marker. Whereas the polar caffeine's (LogD = 0.28 at pH 5) retention differed greatly on the various columns presumably due to the contrasting polarities of the stationary phase which, in turn, would dictate the thickness of the water-ammonium acetate-MeOH adsorbed layer in which caffeine could partition. It was proposed that there was little π-π interaction between phases containing aromatic functionality and caffeine as low retention was observed on these types of phases compared to caffeine's retention on silica. The pK_a values of analytes and that of the stationary phase ligands in SFC CO_2-MeOH mobile phases are unknown [32]. The selectivity of the neutral and acidic components was observed to be independent of the type of stationary phase, with only caffeine exhibiting different retentivity/selectivity due to different degrees of partitioning with the varying polarity stationary phases. The same was true for the bases chromatographed on the basic and neutral stationary phases. The presence of the aqueous layer adsorbed onto the stationary phase appears to minimise any selectivity conferred by the differing stationary phase ligands for analytes possessing the same charge. However, on the acidic phases, the elution order of amitriptyline and propranolol switched around suggesting a change in the dominant retention mechanism (i.e. greater electrostatic attraction on the acidic silica phase).

The basic analytes (i.e. amitriptyline, propranolol and atenolol pK_a values in water = 9.2 - 9.5) all exhibited lower retention on the basic stationary phases (i.e. 2-EP, DEAP, Basic and PA pK_a approximate values of the ligands in water = 5.5 - 10.5) presumably due to ionic repulsion between the protonated bases and the positive character of the stationary phase. If the pH of the SFC mobile phases is approximately 5, then all the basic analytes would be protonated, and the stationary phases would be fully or partially ionised assuming their pK_a's do not change significantly under SFC conditions. In contrast, the basic analytes were retained longer on the two silica phases due to, presumably, ionic attraction between the protonated bases and the ionised silanol groups (pK_a values can range from 2 – 7 depending on the acidity of the silanol groups). Retention on the neutral phases (i.e., cyano and diol) was intermediate due to limited

access of the protonated basic analytes to the ionised silanol groups on the base silica. Interestingly, the 4-EP stationary phase behaved more like a neutral phase even though it possessed a basic functionality.

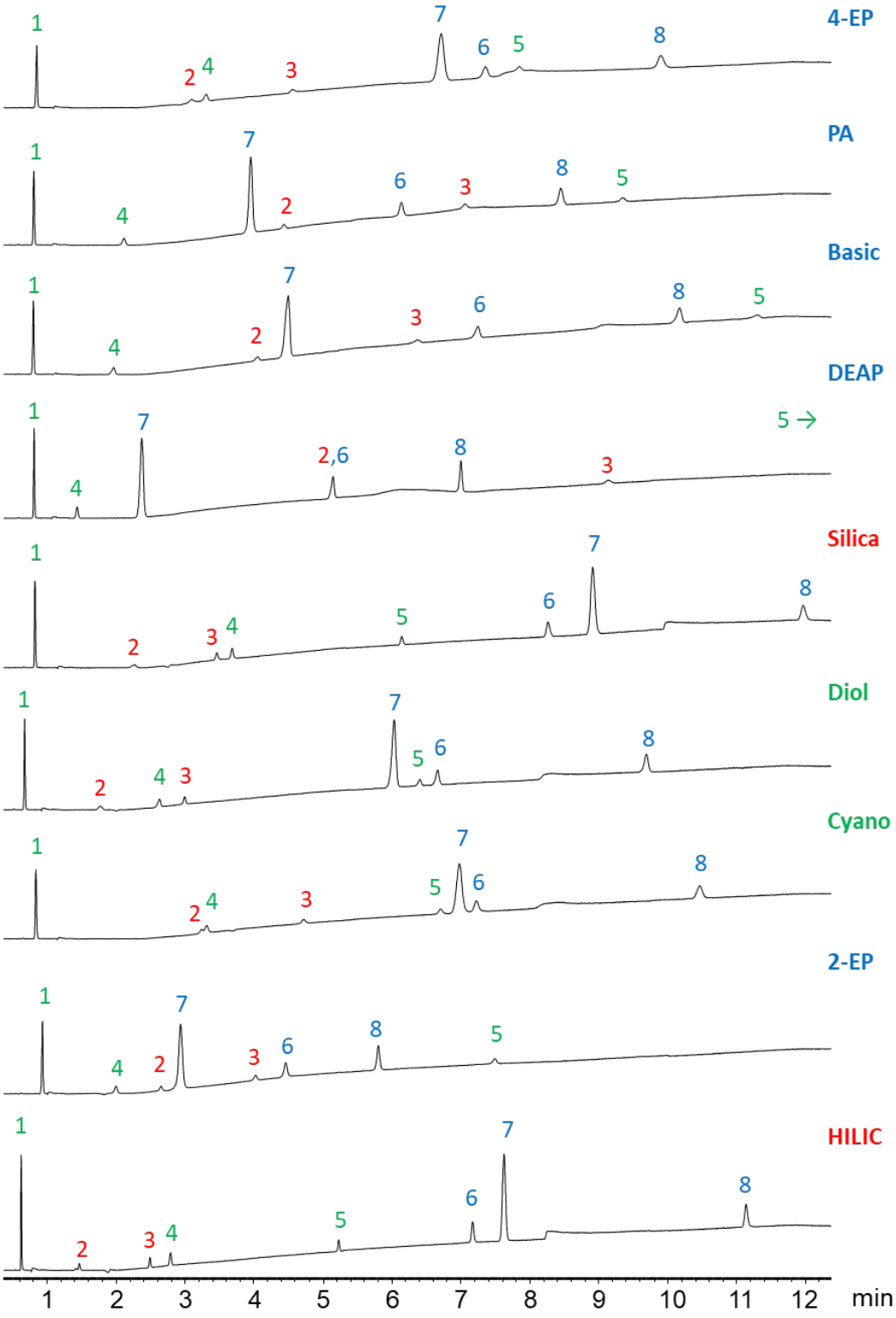

Fig 2. Representative chromatograms of the acid (red), neutral (green) and base (blue) suitability test mixture as a function of differing stationary phase chemistry. Peak assignments: 1 = naphthalene, 2 = ibuprofen, 3 = flurbiprofen, 4 = caffeine, 5 = bendroflumethiazide, 6 = propranolol, 7 = amitriptyline and 8 = atenolol. Generic gradient SFC conditions were employed as described in Section 2.3.

In comparison, the reverse retention behaviour was highlighted for ionised acidic analytes, with low retention on the negatively charged silica phases (electrostatic repulsion) and high retention on the positively charged basic phases (electrostatic attraction). It was noted that on all the stationary phases ibuprofen and flurbiprofen were well separated despite their similarity exhibited in the PCA score plot (see Supplementary Information Fig. S1). Their resolution was enhanced with the strongly basic phases demonstrating the dominance of the electrostatic interaction [34] (Fig. 2).

A measurement of chromatographic selectivity (*S*) differences between the nine SFC phases can be derived from the correlation of the retention time of the eight disparate analytes between differing phases chromatographed using the same SFC conditions. The selectivity correlations were determined using Equation 1.

$$S = 100\ \sqrt{1 - R^2} \qquad \text{Equation 1 [35]}$$

where R^2 = correlation coefficient between the retention times of two phases under the same specified SFC conditions. *S* values of 0 and 100 signify phases of equal and orthogonal retention selectivity.

The results (Table 2) highlight that there were excellent selectivity differences for the acid/neutral/base test mixture between the acidic and basic phases (*S* ranges between 74 – 96), only moderate selectivity differences between the acidic and neutral phases (including the 4-EP, *S* values range between 17 – 36) and there was little difference between the two differing silica phases (*S* values = 7). As expected, there were moderate selectivity differences between the disparate basic phases (excluding the 4-EP, *S* values range between 16 – 87). These results confirm those previously reported [22].

Another way of visualising the data was to determine the individual selectivity factors and analyse these by the chemometric tool of Hierarchical Cluster Analysis (HCA), which illustrates the similarity in selectivity between the two acidic silica phases and the diol (presumably due to access to the silica's surface silanol groups). The neutral CN phase was

shown to be like the 4-EP phase. Comparing the basic phases, the DEAP phase was shown to be more dissimilar to the Basic phase which, in turn, was more dissimilar to the PA and 2-EP phases (Fig. 3).

Table 2. Selectivity values (*S*) on the acidic (red), neutral (green) and basic (blue) columns. *S* value in non-parenthesis and parenthesis are for the range of acidic, neutral and basic components and diphenidine derivatives respectively using the generic gradient SFC conditions described in Section 2.3.

	HILIC	Silica	Cyano	Diol	4-EP	Basic	DEAP	2-EP	PA
HILIC	0	7 (4)	27 (5)	23 (10)	36 (8)	74 (16)	96 (37)	80 (37)	82 (22)
Silica		0	26 (5)	23 (11)	35 (9)	74 (18)	96 (38)	80 (38)	81 (24)
Cyano			0	22 (8)	17 (6)	57 (15)	87 (37)	65 (36)	64 (22)
Diol				0	18 (6)	58 (7)	93 (31)	65 (34)	69 (17)
4-EP					0	47 (11)	87 (34)	54 (34)	57 (19)
Basic						0	62 (28)	16 (32)	57 (13)
DEAP							0	62 (24)	43 (24)
2-EP								0	27 (30)
PA									0

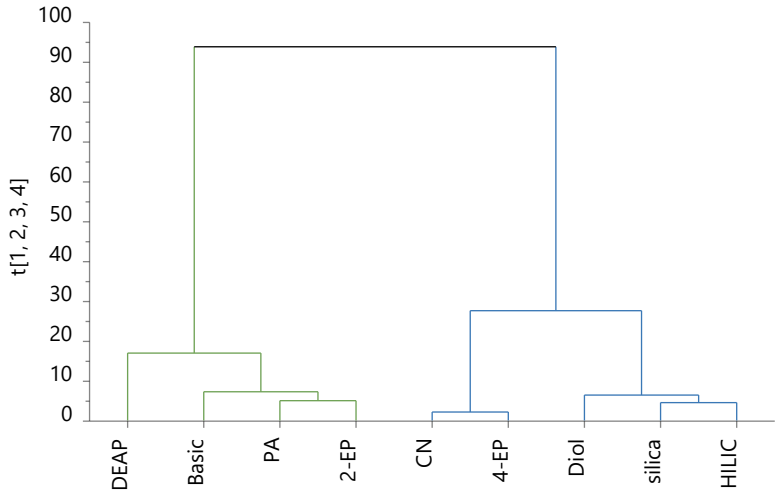

Fig. 3. Hierarchical cluster analysis of the SFC stationary phases analysed using the acid/ neutral/base suitability test mixture

These selectivity differences can be observed visually in the chromatograms shown in Fig. 2. The eight phases from the same manufacturer yielded acceptable tailing factors for all analytes

(i.e. mean T_f values of 1.02, *SD* = 0.14, 0.95, *SD* = 0.07 and 0.93, *SD* = 0.08 for the acidic, neutral and basic phases respectively).

3.2 Generic SFC gradient chromatographic separation of regioisomeric diphenidines

The range of acidic, neutral and basic columns (see Table 1) was selected to evaluate the chromatographic selectivity of the diphenidines. The diphenidines are basic (calculated pK_a range in water = 8.3 – 9.4), however, their ionisation state in supercritical carbon dioxide-MeOH mixtures is uncertain [32]. A simple 5 - 40% linear CO_2-MeOH gradient using an aqueous ammonium acetate buffer incorporated into the MeOH successfully chromatographed all 31 diphenidine analogues on the nine differing SFC stationary phases using the generic gradient SFC conditions described in Section 2.3.

3.2.1 Effect of stationary phase on the retentivity

The LogD values for the library of diphenidines (**1 – 31**) ranged from 1.06 – 2.87 at pH 5 [10], however all eluted within the gradient time cycle time of 12 minutes (i.e. t_R min = 0.85 min and t_R max = 8.13 min, see Supplementary Information Tables S1 – S8) compared to the void volume marker naphthalene (LogD = 3.37, t_R mean = 0.79 min). Certain diphenidines (i.e. **5**, **6** and **7**, LogD = 2.15 – 2.32) exhibited low retention but the methodology still separated these early eluting regioisomers (see Fig. 4). LogD values of a simplified subset of the *meta*- and *para*-substituted diphenidine regioisomers (**3**/**4**, **6**/**7**, **15**/**16**, **18**/**19**, **21**/**22**, **24**/**25**, **27**/**28** and **30**/**31**) were found to be poorly correlated to the logarithm of their retention times, however, reasonable correlation was observed against their Hammett substituent constants (r^2 = 0.82, 0.83 and 0.68 respectively) for acidic (i.e. silica), neutral (i.e. diol-) and basic (i.e. 2-EP) phases. The lower correlation for the 2-EP phase was attributed to the larger errors associated with lower retention. The retention order of this subset of diphenidines was found to be independent of the nature of the stationary phase suggesting a common retention mechanism was occurring on the acidic, neutral and basic phases. A plausible explanation is that the diphenidines possessing electron-donating substituents are more likely to partition into the adsorbed aqueous layer surrounding the stationary phase in comparison to those possessing electron withdrawing substituents. All the diphenidine derivatives exhibited lower retention on the basic phases which can be attributed to the repulsion of the protonated diphenidines by the protonated ligands on the stationary phase.

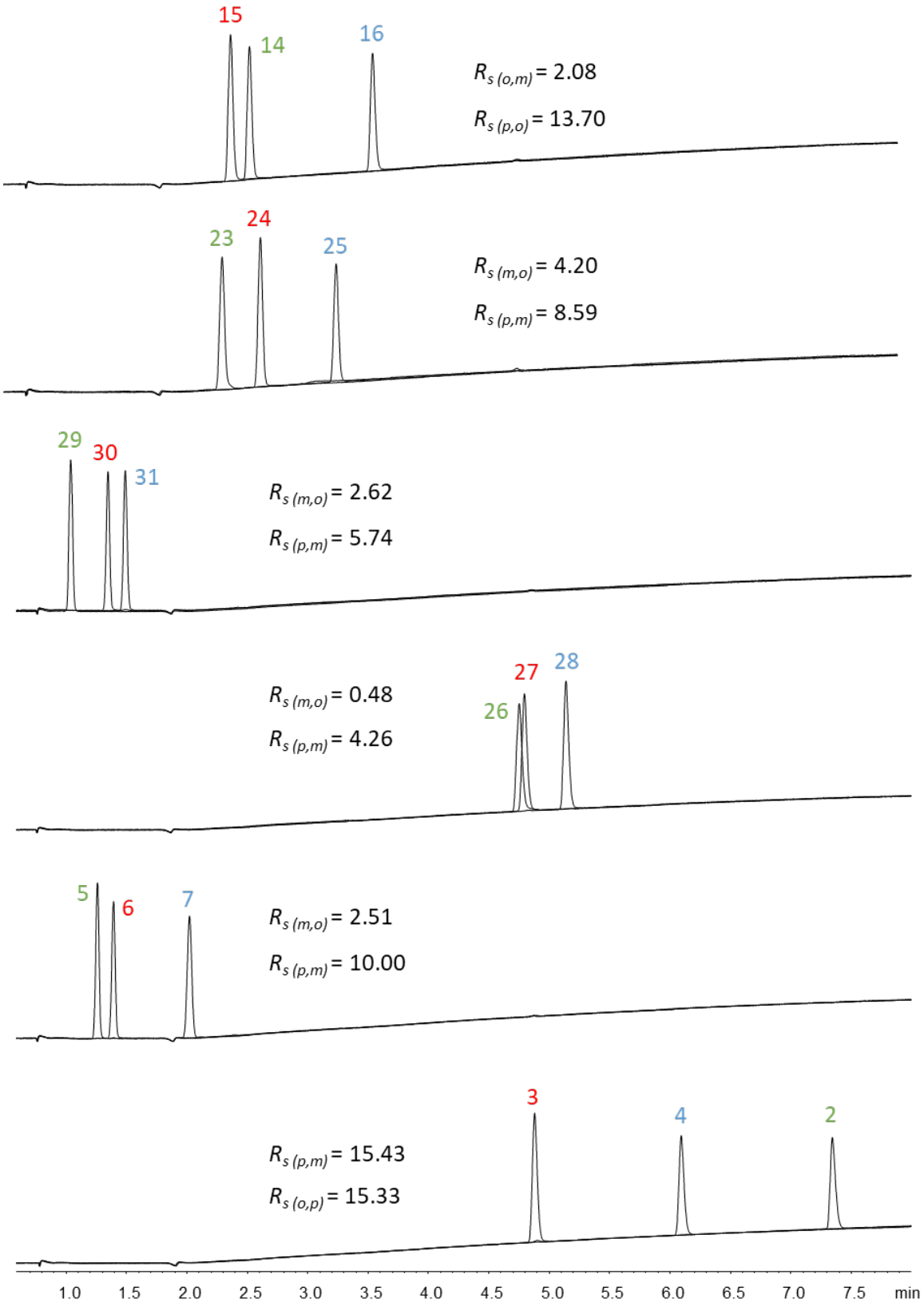

Fig. 4. Representative chromatograms for a range of substituted diphenidine regioisomers (*ortho*-, green; *meta*-, red and *para*-, blue) analysed on the HILIC silica column. Generic gradient SFC conditions were employed as described in Section 2.3.

Retention was greatest on the silica phases, presumably due to electrostatic interaction with the ionised silanol groups in addition to partitioning into the adsorbed aqueous layer whose thickness was dependent on the nature of the bonded ligand. The retention of the diphenidines on the neutral phases (i.e. cyano- and diol-) was intermediate. The eight phases from the same manufacturer yielded acceptable peak tailing factors (i.e., mean T_f values of 1.04, SD = 0.05, 0.92, SD = 0.05 and 0.93, SD = 0.07 for the acidic, neutral and basic phases respectively) irrespective of the ligand chemistry.

3.2.2 Effect of stationary phase on the selectivity of diphenidines

The S values for the SFC analysis of 31 diphenidine derivatives on the nine different stationary phases (see Table 2) highlighted similarities between the two silica phases despite one being fully and the other superficially porous in nature (S value = 4), the cyano-, diol- phases and 4-EP phases (S value = 5-11), whereas the basic phases (DEAP, 2-EP and PA) were observed to be more dissimilar (S value = 13-38). The greatest selectivity differences were observed with the acidic and basic phases (S value = 16-38) and the smallest between acidic and neutral phases (S value = 5-11).

3.2.3 Separation of substituted regioisomeric diphenidines

Baseline separation of the diphenidine regioisomers (**2 – 4**, **5 – 7**, **14 – 16**, **17 – 19**, **20 – 22**, **23 – 25**, **26 – 28** and **29 - 31**) was achieved for six of the eight groups of substituted derivatives evaluated. The *ortho*- and *meta*-regioisomers of bromo- and methyl-derivatives (i.e., **20**/**21** and **26**/**27** respectively) were only partially separated (Fig. 4 and Fig. 5). As the size of the halogenated substituent increased, the separation of the *ortho*- and *meta*-isomers decreased, resulting in near co-elution for **20** and **21**. In comparison, good separation was achieved with the larger iodo-substituted analogues (**23** – **25**) and a switch in elution order (for the fluphenidines, **14** – **16**, and chlorodiphenidines, **17** – **19**, the elution order was *meta*- < *ortho*- < *para*-, whereas, with **23** – **25** the elution order was observed to be *ortho*- < *meta*- < *para*- for the substituted regioisomers (Fig. 5).

It has been previously reported that the SFC elution order of the fluoro-, chloro- and bromo-amphetamine (and methamphetamine) regioisomers using a diol-column and mobile phase of ammonia-MeOH was observed to be *ortho*- < *meta*- < *para*-substituted regioisomer [13]. The elution order of the fluoro-, chloro- and bromo-amphetamine and methamphetamine regioisomers differed from that observed with the corresponding diphenidines. This was not unexpected given that the steric accessibility of the amphetamine (primary nitrogen) and

methamphetamine (secondary nitrogen) regioisomers would be different from the diphenidines (tertiary nitrogen).

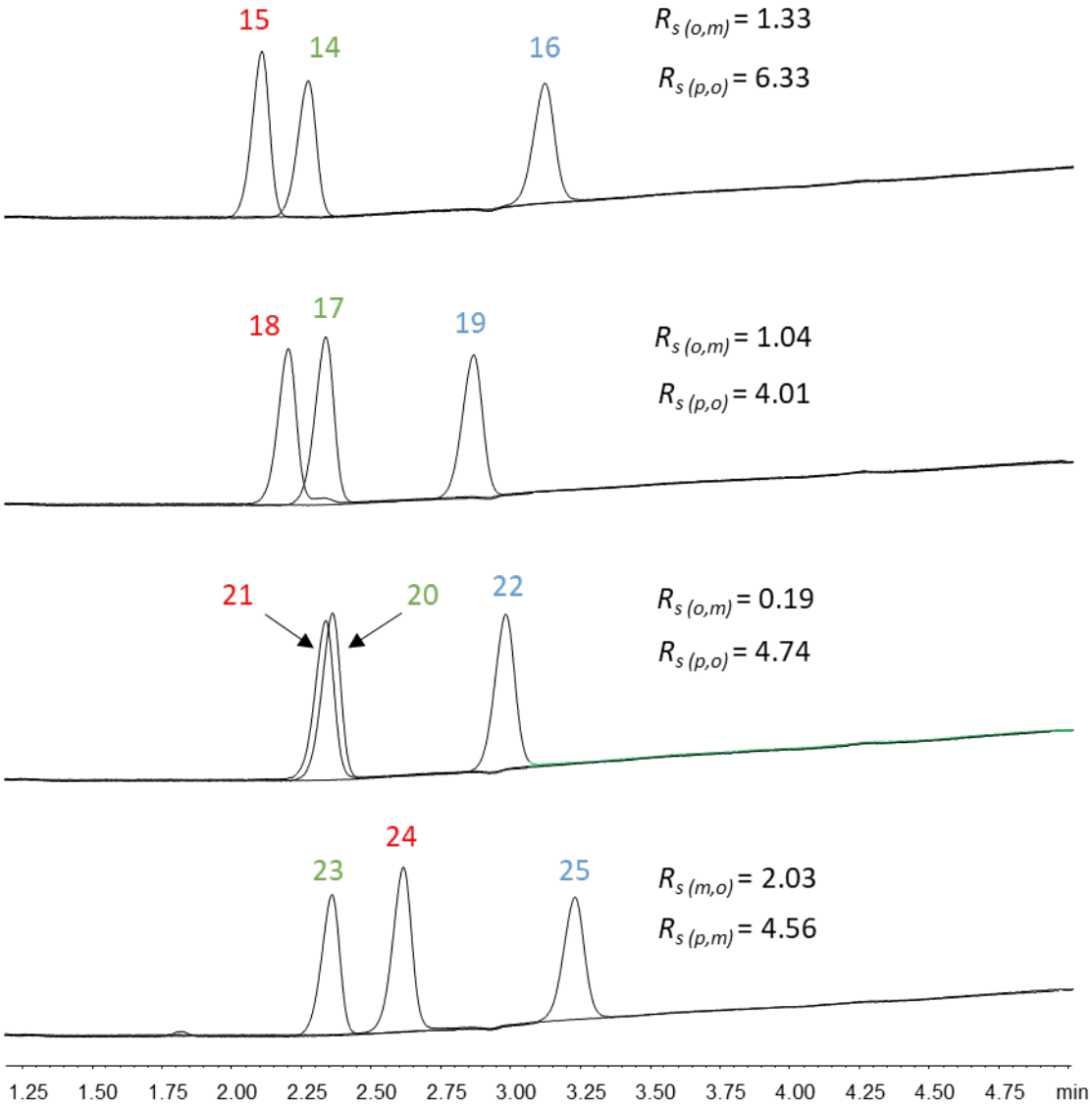

Fig. 5. Representative chromatograms for halo-substituted diphenidine regioisomers (*ortho*-, green; *meta*-, red and *para*-, blue) analysed on the 4-EP column. Generic gradient SFC conditions were employed as described in Section 2.3.

The ligand chemistry of the stationary phases did not appear to affect the elution order of the individual 2-, 3- and 4-methoxphenidines, **2** – **4** (Fig. 6), even though we had previously demonstrated that the stationary phases were highly complementary to one another for the acid/neutral/base suitability test mixture (see Table 2). This phenomenon was also observed for the analysis of diphenidine derivatives by RP-UHPLC where electrostatic interaction was shown to be the predominant retention mechanism and masked the more subtle reverse-phase

interactions [9, 10]. This suggested that partitioning into the aqueous layer surrounding the stationary phases, as well as the electrostatic interactions between the protonated diphenidine derivatives and the ionised silanol groups, may be the primary retention mechanisms under these SFC conditions [27, 34].

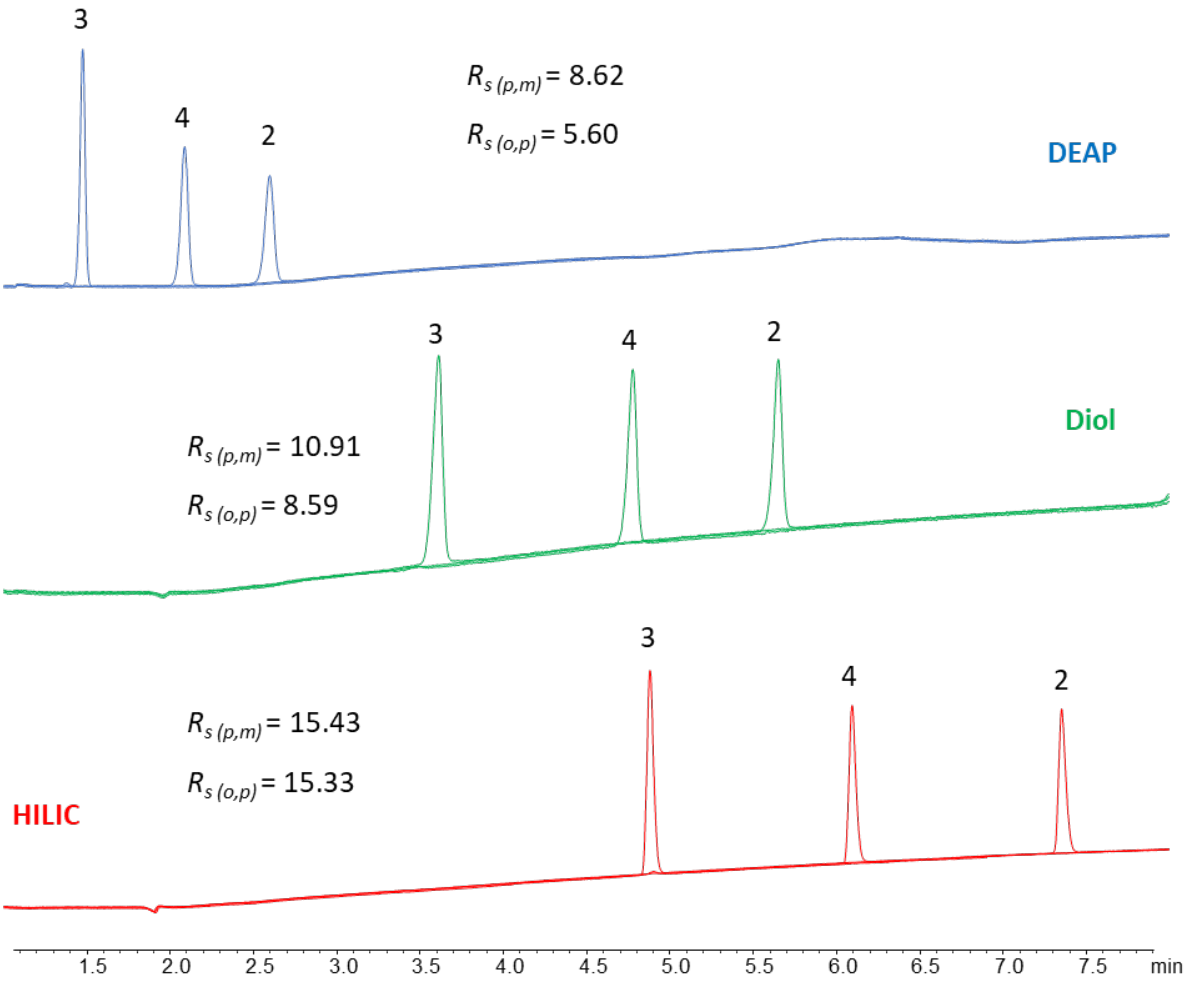

Fig. 6. Representative chromatograms for methoxphenidines (**2**, **3** and **4**) analysed on acidic (red), neutral (green) & basic (blue) columns. Generic gradient SFC conditions were employed as described in Section 2.3.

Interestingly, the 2,3-(methylenedioxy)diphenidine (**8**) and (2,2-difluoro-1,3-benzodiox-4-yl)diphenidine (**11**) both eluted before their corresponding structural isomers 3,4-(methylenedioxy)diphenidine (**9**) and (2,2-difluoro-1,3-benzodiox-5-yl)diphenidine (**12**) respectively on all the phases evaluated; in all cases excellent resolution was achieved (R_s (**8**, **9**) ranged from 2.34 to 10.86 [mean = 5.49] and R_s (**11**, **12**) ranged from 1.95 to 15.85 [mean = 6.56]), except for the 2-EP stationary phase which only afforded partial separation of **11** and **12**.

The elution order of the diphenidine regioisomers of the various mono-substituted derivatives was not the same in all cases and may be dependent on subtle differences in both the size and accessibility of the specific regioisomer into the stationary phase rather than the substituent's electron-donating or withdrawing capacity. For example, the electron-withdrawing substituted derivatives (i.e., **14** – **16** versus **5** – **7** and/or **29** – **31**) generated differing elution orders (see Fig. 4). The trifluoromethoxy- and trifluoromethyl- elution pattern (*ortho-* < *meta-* < *para*) was similar to that of the large iodo-substituted analogues (**23** – **25**). Interestingly, the electron-donating methyl- (i.e., tolphenidines, **26** – **28**) and electron-withdrawing iodo-derivatives (**23** – **25**) both yielded similar elution profiles.

3.3 Comparison between generic gradient RP-LC and SFC

An orthogonal or complementary elution order was observed between the RP-UHPLC and SFC stationary phases (see Supplementary Information Fig. S2 – S4). Generic UHPLC was performed on an ACE Excel C18 (1.7 µm, 100 Å, 100 x 3 mm i.d.) column using a 10 mM aqueous ammonium acetate (unadjusted pH, approx. pH 6.5) and 10 mM aqueous ammonium acetate in water / MeCN (2:8 v/v) gradient (t_G = 10 minutes) at 60°C and 0.65 mL min^{-1} flow rate. In general, hydrophobic compounds which were highly retained on a reverse phase column were less retained by SFC. Most of the diphenidines possessed high LogD values [10] suggesting poor retention on SFC columns, however, all the diphenidines were retained to certain degrees. Selectivity values ranged from 35 – 59 (the 2-EP and DEAP being the most different) for the nine SFC phases compared against RP-UHPLC using a high purity silica C18 phase (see Supplementary Information Fig. S2 – S4 and Table S9) highlighting the complementary nature of both separation techniques. The general elution order of the halogenated regioisomers (**14** – **16**, **17** – **19**, **20** – **22** and **23** – **25**) using RP-UHPLC conditions was observed to be *para-* < *ortho-* < *meta-* for the substituted regioisomers. However, as the size of the halogenated substituent increased, the *meta*-isomer retention decreased, leading to a switch in the elution order for the iododiphenidines (i.e., *para-* (**25**) < *meta-* (**24**) < *ortho-* (**23**) elution order) compared to the smaller fluoro-substituted analogues (i.e. *para-* (**16**) < *ortho-* (**14**) < *meta-* (**15**) elution order) [10]. In comparison, with SFC the general elution order was observed to be *meta-* < *ortho-* < *para-* for **14** – **16**, **17** – **19**, **20** – **22** and **23** – **25** but as the size of the halogenated substituent increased the *meta*-regioisomers was retained longer, once again resulting in an elution order switch with the iododiphenidines (i.e. *ortho-* (**23**) < *meta-* (**24**) < *para-* (**25**) elution order) compared to fluphenidines (i.e. *meta-* (**15**) < *ortho-* (**14**) < *para-* (**16**) elution order). The cases where the generic SFC methodology failed to separate

the *ortho, meta and para* isomer of the substituent diphenidines (i.e., **20**/**21** and **26**/**27**) the generic UHPLC methodology provided baseline separation. *Vice versa,* when the generic UHPLC methodology failed to deliver baseline separation (i.e., **14**/**15**, **17**/**18** and **30**/**31**) the generic SFC methodology did.

4.0 Conclusion

A range of acidic, neutral and basic SFC stationary phases have been shown by selectivity correlations and Hierarchical Cluster Analysis (HCA), to exhibit complementary chromatographic selectivity towards an acid/neutral/base suitability test mixture. The retentivity of basic analytes towards stationary phases followed the order acidic > neutral > basic. The reverse order of retentivity was observed with acidic analytes. The results infer that analytes partition into the aqueous pseudo stationary phase layer depending on their polarity. Once in the aqueous layer the protonated basic analytes interact *via* electrostatic attraction with the ionised silanol groups or *via* electrostatic repulsion with protonated species on the stationary phases. The reverse is observed with acidic analytes, retention of neutral analytes appears to be dedicated by their polarity and partitioning into the aqueous layer. The results highlight the importance of screening acidic (i.e. silica), neutral (i.e. cyano-) and basic (i.e. DEAP and 2-EP) stationary phases for the SFC analysis of unknown mixtures. All the phases generated acceptable tailing factors irrespective of the analyte's physical and/or chemical properties and stationary phase ligand chemistry.

A simple linear generic gradient SFC methodology employing ammonium acetate and water in the MeOH has been shown to be highly suitable for the rapid analysis (<10 mins) of a library of 31 novel 1,2-diarylethylamines (also known as diphenidine analogues). Medium to large selectivity differences were exhibited between the acidic, neutral and basic SFC phases and the regioisomeric diphenidines which differed only in their substituent position on the aromatic ring. The greatest selectivity differences were observed with the acidic and basic phases and the smallest between acidic and neutral phases. The presence of the water-ammonium acetate-MeOH pseudo-stationary phase layer appears to minimise the more subtle selectivity differences conferred by the differing ligands. Once adsorbed onto the polar stationary phase, the water-ammonium acetate-MeOH layer promotes partitioning of the protonated diphenidines into the adsorbed aqueous layer where they can participate in further electrostatic attraction (i.e. silica resulting in longer retention) or repulsion (i.e. basic phases resulting in short retention) depending on the ionisation state of the stationary phase. The retention of the

diphenidines on the neutral phases (i.e. cyano- and diol-) was intermediate. For individual substituted *ortho*-, *meta*- and *para*-isomers, the same elution order was observed irrespective of the nature of the stationary phase. SFC effected acceptable separation of the *ortho*-, *meta*- and *para*-isomers of six of the eight families of analogues evaluated. As the size of the halogen substituent increased, the SFC separation of the *ortho*- and *meta*-isomers decreased, resulting in co-elution for the 2- and 3-bromodiphenidines (**20**/**21**). The smaller fluoro- (fluphenidines, **14** - **16**) and chloro-substituted derivatives (**17** – **19**) generated an elution order of *meta*- < *ortho*- < *para*- for the substituted isomers, whereas, with the iodophenidines (**23** – **25**) the elution order was *ortho*- < *meta*- < *para*- under SFC conditions. This contrasted with RP-UHPLC where the elution order for the fluphenidines and iodophenidines was *para*- < *ortho*- < *meta*- and *para*- < *meta*- < *ortho*- respectively. The elution order of the regioisomeric diphenidine derivatives was observed not to be the same in all cases and may be dependent on subtle unexplained retention mechanisms.

An orthogonal elution order of the analytes was demonstrated between the RP-UHPLC and SFC stationary phases due to the polarity differences between the separation modes. In general, hydrophilic compounds which were poorly retained on a C18 reverse phase column were well retained on SFC columns thus permitting the analyst to choose the most appropriate mode of chromatography to facilitate good retention which is especially important when the chromatographic technique is coupled to MS detection [26, 29].

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6.0 Declaration of Interests

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.

7.0 Author Contributions

Graeme Cochrane: Methodology, Formal analysis, Investigation.

Jennifer K. Field: Methodology, Formal analysis, Investigation.

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Ryan E. Mewis: Methodology, Formal analysis, Investigation, Supervision.

Melvin R. Euerby: Conceptualization, Methodology, Formal analysis, Writing - review & editing, Supervision.

Oliver B. Sutcliffe: Conceptualization, Methodology, Writing – review & editing, Supervision.

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