Reversed-phase chiral HPLC and LC/MS analysis with tris(chloromethylphenylcarbamate) derivatives of cellulose and amylose as chiral stationary phases

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ABSTRACT

Three polysaccharide-derived chiral stationary phases (CSP) were evaluated for the resolution of more than 200 racemic compounds of pharmaceutical interest in the reversed-phase (RP) separation mode. The population of test probes was carefully evaluated in order to insure that it covers as completely as possible all structural diversity of chiral pharmaceuticals. RP showed the highest potential for successful chiral resolution in HPLC and LC/MS analysis when compared to normal phase and polar organic separation modes. Method development consisted of optimizing mobile phase eluting strength, nature of organic modifier, nature of additive and column temperature. The newer CSPs, cellulose tris(3-chloro-4-methylphenylcarbamate) and amylose tris(2-chloro-5-methylphenylcarbamate), were compared to the commonly used cellulose tris(3,5-dimethylphenylcarbamate) in regards to their ability to provide baseline resolution. Comparable success rates were observed for these three CSPs of quite complimentary chiral recognition ability. The same method development strategy was evaluated for LC/MS analysis. Diethylamine as additive had a negative effect on analyte response with positive ion mode electrospray (ESI$^+$) MS(/MS) detection, even at very low concentration levels (e.g., 0.025%). Decreasing the organic modifier (acetonitrile or methanol) content in the mobile phase often improved enantioselectivity. The column temperature had only a limited effect on chiral resolution, and this effect was compound dependent. Ammonium hydrogencarbonate was the preferred buffer salt for chiral LC with ESI$^+$ MS detection for the successful separation and detection of most basic pharmaceutical racemic compounds. Ammonium acetate is a viable alternative to ammonium hydrogencarbonate. Aqueous formic acid with acetonitrile or methanol can be successfully used in the separation of acidic and neutral racemates. Cellulose tris(3-chloro-4-methylphenylcarbamate) and amylose tris(2-chloro-5-methylphenylcarbamate) emerge as CSPs of wide applicability in either commonly used separation modes rivaling such well established CSPs as cellulose tris(3,5-dimethylphenylcarbamate). Screening protocols including these two new CSPs in the preferentially screened set of chiral columns have higher success rates in achieving baseline resolution in shorter screening time.

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1. Introduction

Chiral reversed-phase (RP) HPLC and LC/MS methods of analysis are primarily developed for applications targeting (polar) compounds poorly soluble in alkanes and low molecular weight (MW) alcohols or mixtures thereof [1] and for bioanalytical applications.

Hydrogen bonding interactions are considered to be essential to chiral recognition with polysaccharide-based chiral stationary phases (CSP) [2]. Such interactions are expected to take place between analyte molecules and the CSP, mainly in the absence of strongly competing species such as water. Therefore, chiral separations on polysaccharide-based CSPs (the most widely used class of CSP) are primarily explored in the normal phase (NP) separation mode using mixtures of alkanes (e.g., hexane) and low MW alcohols as mobile phase (MP). Additional separation modes use sub- or super-critical fluids or polar organic solvents (acetonitrile or alcohols) as MP. Nevertheless, chiral recognition is possible even under conditions unfavorable for hydrogen bonding between analytes and CSP as proved with the first RP method using polysaccharide-derived CSPs reported by Ikeda et al. [3]. Ever since, analysts have explored RP chiral HPLC regarding various considerations such as sample origin, analyte solubility and/or required sensitivity, the latter necessitating the use of mass spectrometry as method of detection [4–8].

To support drug metabolism and pharmacokinetic studies of chiral pharmaceuticals, it is necessary to combine the resolving power of HPLC with the sensitivity of mass spectrometric detection. Chiral LC/MS methods must be selective, fast (effective in high throughput laboratories), robust and sensitive to low levels of distomer in the presence of the eutomer; they must also be free of interferences from matrix components present in complex biological samples such as blood, tissue and urine. The speed of chiral LC/MS(/MS) analysis is primarily dependent on the separation efficiency of the chiral HPLC column. Advancements in column technology have made higher efficiency CSPs available for routine chiral separations [9,10]. As long as the enantiomers of interest to an assay are chromatographically resolved, further selectivity *may not* be required on part of the CSP due to the unique specificity of MS/MS detection, which allows for the simultaneous quantification of a parent drug and its metabolites in samples of biological origin. This specificity reduces the need for complete chromatographic resolution of all species of interest, leading to shorter analysis times and increased sample throughput.

Chiral RP LC applications using polysaccharide-based CSPs were reviewed by Tachibana and Ohnishi [11]. They listed close to 100 compounds reported to have been successfully resolved in both RP and some other chiral separation mode (NP or polar organic (PO)), or exclusively in RP. Hence, RP emerges as an alternative to other separation modes both when it proves to be similar or complimentary in selectivity for particular separation challenges. Most often, chaeotropic reagents are used as MP additives in RP (chiral) LC for improved resolution. Some examples are potassium hexafluorophosphate, sodium perchlorate, potassium tetrafluoroborate, sodium dihydrogenphosphate, sodium tetraborate or phosphoric acid [5]. Unfortunately, all these reagents are non-volatile, hence incompatible with atmospheric pressure ionization (API) MS. Developing fast and sensitive chiral LC separations compatible with mass spectrometric detection has remained a challenge to analysts.

The most widely used polysaccharide-based CSPs are cellulose tris(3,5-dimethylphenylcarbamate), amylose tris(3,5-dimethylphenylcarbamate) and cellulose tris(4-methylbenzoate) [12–15]. These CSPs demonstrate wide chiral recognition ability, good chemical stability and high loadability in all common separation modes: NP, PO, supercritical fluid (SFC) and RP chiral LC [15]. These CSPs differ in their utility in separating chiral compounds at large, with amylose and cellulose tris(3,5-dimethylphenylcarbamate)s being the most successful ones [12–14,16]. Current screening protocols targeted at identifying combinations of CSP/MP conditions capable of providing adequate resolution can be dramatically improved by including chloromethylphenylcarbamates of cellulose and amylose in the preferentially screened set of chiral columns [9,17–19].

The tris(halomethylphenylcarbamate) derivatives of cellulose and amylose were first proposed and evaluated as chiral selectors in HPLC by Chankvetadze et al. [20–23]. Such CSPs were first made commercially available under the brand name Sepapak (Sepaserve, Muenster, Germany) and more recently as Lux chiral HPLC columns (Phenomenex, Torrance, CA, USA). Sepapak and Lux columns have been the focus of several investigations conducted in NP, PO or RP separation modes [9,17,24,25]. Lux Cellulose-2 (cellulose tris(3-chloro-4-methylphenylcarbamate)) and Lux Amylose-2 (amylose tris(2-chloro-5-methylphenylcarbamate)) demonstrate wide chiral recognition ability, similar to the aforementioned dimethylphenylcarbamate derivatives, as well as significant complementarity in all commonly used separation modes. Similar conclusions were reached in a recent study comparing the selectivity of cellulose and amylose tris(chloromethylphenylcarbamate) derivatives to other polysaccharide-based CSPs in NP and PO [19].

To our knowledge, no comprehensive evaluation of the performance of cellulose and amylose tris(chloromethyl-phenylcarbamate) derivatives in reversed-phase LC has been published to date. Zhou et al. recently published a study on various CSPs, including cellulose tris(3-chloro-4-methylphenylcarbamate), using a small number of *neutral* racemates as test probes in NP and RP [25]. Also, these CSPs have not been compared to the commonly used cellulose and amylose tris(dimethylphenylcarbamate)s in regards to their success in resolving racemic mixtures at large.

In this work we explore the performance of cellulose tris(3-chloro-4-methylphenylcarbamate) and amylose tris(2-chloro-5-methylphenylcarbamate) CSPs and compare them to cellulose tris(3,5-dimethylphenylcarbamate) in regards to ability to resolve racemic compounds in reversed-phase mode using MPs compatible with MS and/or UV detection. These CSPs were screened in a number of RP MPs for the separation of over 200 racemates (mostly generic APIs). Performance was evaluated based on success rates in *baseline* resolving members of this diverse group of chemical compounds. The intense screening effort serving as basis for this report resulted in clear patterns that lend themselves to devising expeditive screening protocols and method optimization with chloromethylpehnylcarbamates of cellulose and amylose as CSPs.

2. Experimental

2.1. Chemicals and reagents

HPLC-grade acetonitrile, methanol and water were purchased from Burdick & Jackson (Morristown, NJ, USA). ACS-grade formic acid, acetic acid, ammonium acetate, ammonium hydrogencarbonate and diethylamine (DEA) were obtained from Sigma–Aldrich (St. Louis, MO, USA). All screened racemates were obtained from Sigma–Aldrich, except for benzodiazepines and ketamine and its derivatives, which were purchased as 1.0 mg/ml stock solutions from Cerilliant.

Stock solutions of racemic compounds were prepared in methanol at a concentration of 1–5 mg/ml (depending on detector response) and diluted to 100–500 μg/ml for UV detection and 500 ng/ml for MS detection. The injection volume was 1–5 μl depending on detector response.

2.2. Instrumentation

An Agilent HP1100 liquid chromatography system (Agilent Technologies, Palo Alto, CA, USA) was used for chromatographic separations. A Synergi column selector (Phenomenex, Torrance, CA, USA) accommodating 6 HPLC columns was used with good success for expediting chiral screening.

Chiral chromatographic separations followed by UV detection were performed using Lux Cellulose-1, Lux Cellulose-2 and Lux Amylose-2 HPLC columns with the dimensions 250 mm × 4.6 mm i.d. packed with 5 μm particles (Phenomenex, Torrance, CA, USA). Typically faster LC/MS analyses were performed on columns with the dimensions 150 mm × 2.1 mm i.d. packed with 3 μm particles (same source). The acidic MP consisted of 0.1% acetic, formic, or trifluoroacetic acid in water (solvent A) and acetonitrile or methanol (solvent B). It was used for resolving acidic or neutral racemates. Ammonium hydrogencarbonate (5–20 mM) or ammonium acetate (5–20 mM), with or without the addition of 0.1% DEA (solvent A) in mixture with acetonitrile or methanol (solvent B), was used for separating basic or neutral racemates. All chiral separations were attempted in isocratic elution mode at room temperature at flow rates of 1.0 or 0.2 ml/min with UV or MS detection, respectively, and in MP of various eluting strength. The UV detector was set at 220 nm.

An API 3000 triple quadrupole mass spectrometer equipped with the TurboIonSpray interface (Applied Biosystem/Sciex, Foster City, CA, USA) was used for all chiral LC–MS/MS experiments reported here. The mass spectrometer was operated in positive ion mode with multiple reaction monitoring (MRM). The dwell time was set to 200 ms for all analytes. The ion source temperature was set to 400 °C and the TurboIonSpray nitrogen flow (drying gas) to 5000 ml/min. Instrument tuning was performed with the Analyst Autotune function followed by ion source optimization by flow injection (FIA) at a flow rate of 200 µl/min in 0.1% formic acid in acetonitrile:water 1:1 (v/v).

Data acquisition and processing were carried out with the ChemStation Rev.A.10.02 software for HPLC (UV) separations and the Analyst 1.4.1 software (Applied Biosystem/Sciex) for LC/MS/MS separations.

3. Results and discussion

The various factors identified in the literature affecting chiral resolution are the nature of chiral compound and of chiral selector, MP conditions and column efficiency and temperature. The effect of these factors on the enantiomer recognition ability of cellulose tris(3-chloro-4-methylphenylcarbamate) and amylose tris(2-chloro-5-methylphenylcarbamate) CSPs in comparison to that of cellulose tris(3,5-dimethylphenylcarbamate) (see chemical structure of each CSP represented in Fig. 1) in reversed-phase mode using MPs compatible with MS and/or UV detection is discussed below.

3.1. Selection of test probes

The fairly large collection of racemic compounds used as test probes in this study (209 different compounds) was assembled randomly, except for their chemical nature. The ratio of acidic to neutral to basic compounds part of this group loosely followed the frequency of the same in current pharmaceutical use. Numerous strong and weak bases and acids, as well as polar and hydrophobic species were included to reflect the diversity of drug compounds under current development. No attempt was made to assess the diversity of this group based on structural descriptors relevant to chirality. Nevertheless, this collection included most of the generic compounds studied by Borman et al., which have been shown to be chemically diverse [12]. Unfortunately, in the current practice of chiral screening, test compounds are selected randomly, often in only small numbers and without a clear concept or justification (e.g., to be representative of racemic compounds at large). In spite of this questionable approach, broad conclusions are drawn on CSP applicability and order of utility [24,26].

To our knowledge, the work published by Borman et al. [12] is the only attempt to date to characterize the population of test compounds used in the determination of the utility of (and order of utility within) a set of preferentially screened chiral HPLC columns. The large population of test compounds used in their study was assessed with principal component analysis (PCA) for diversity based on structural descriptors relevant to chirality. Their analysis showed that *trans*-stilbene oxide, cypermethrin and diperodon delineate a space within the PCA scores plot populated fairly evenly by both generic and proprietary racemic compounds included in this population. The extreme position of *trans*-stilbene oxide on the PCA scores plot is in good agreement with previous experimental results obtained on polysaccharide-derived CSPs. The ease of resolving its enantiomers in most CSP/MP systems has arguably elevated it to the status of most commonly used chiral test probe. Whereas diperodon seems less interesting (placing closer to the main cluster of test compounds, hence apparently being similar to
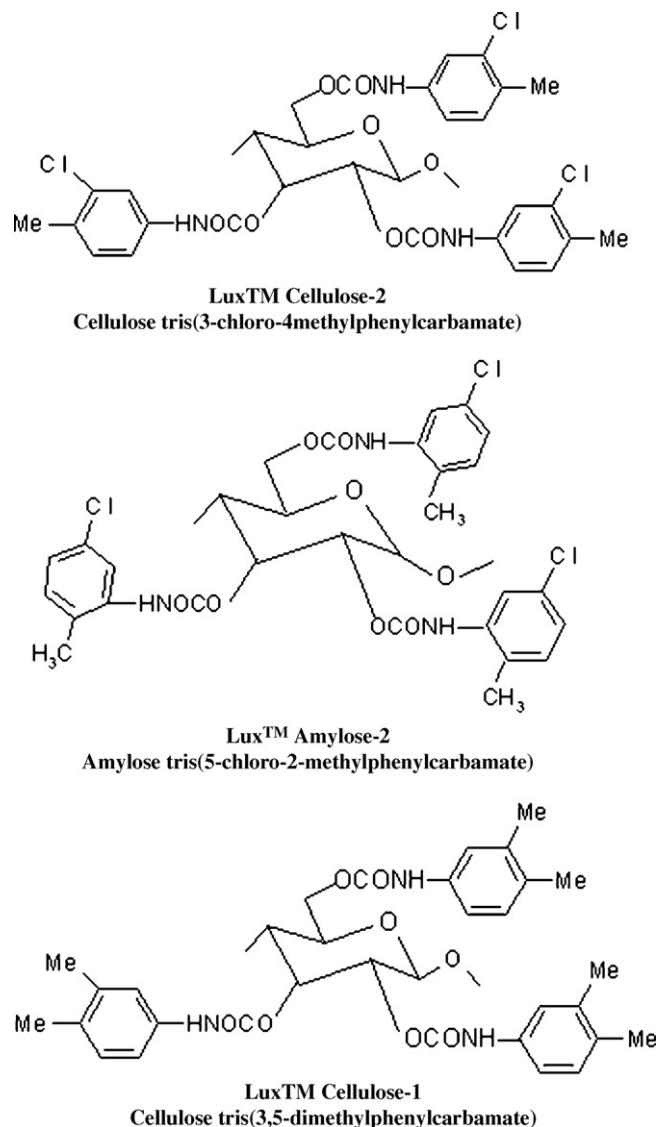

Fig. 1. Chemical structures of the three CSPs evaluated in this study.

other compounds in the pool in terms of PCA scores), cypermethrin does stick out as unusual. Its placement on the plot seems as extreme as that of *trans*-stilbene oxide, but based on different principal components. Its behavior should be interesting to follow in the current study.

The group of generic compounds assessed by Borman et al., for its diversity deserves special attention. Fair correlation was found between the behavior of its members in the various LC systems explored and their relative location on the PCA scores plot [27]. By including most of the generic test compounds analyzed by Borman et al. in the population investigated in the current study, we consider to have ensured proper diversity (additionally, the sheer size of this population may also promote diversity). Another important feature of any population of test compounds is its degree of redundancy, which raises the question of weather a disproportionate presence of groups of test probes of similar behavior may compromise statistical interpretation of results and the conclusions drawn thereupon.

3.2. Effect of organic modifier

The mechanism of enantiorecognition in aqueous/organic MPs made up of a buffer solution mixed with acetonitrile and/or
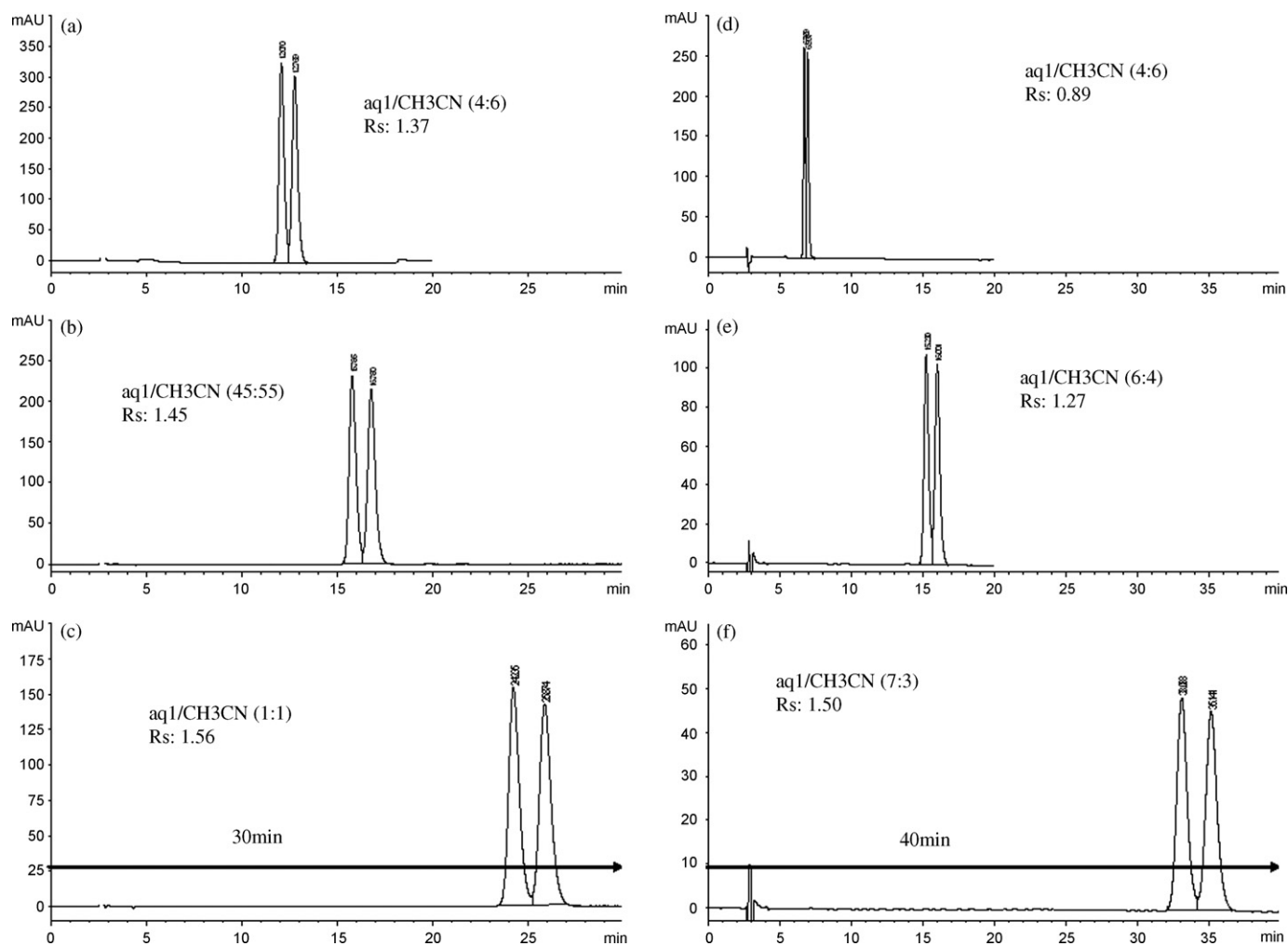
Fig. 2. Effect of mobile phase eluting strength on the retention and chiral resolution of trimipramine (a–c) and ketamine (d–f). Mobile phase aqueous component: aq1 – 20 mM ammonium acetate + 0.1% DEA; column – Lux 5 μm Cellulose-2, 250 mm × 4.6 mm i.d.; flow rate – 1 ml/min; UV detection – 220 nm.

methanol is the result of a combination of various strong and weak interactions taking place between analyte molecules and the chiral stationary phase [2,28]. In addition to hydrogen bonding, π–π, dipole–dipole stacking and steric interactions, hydrophobic interactions between analytes and CSP play an important role. Such interactions are sensitive to the organic component of the aqueous/organic MP, very much like in achiral RP HPLC. By increasing the organic content of the MP, its eluting strength increases, the hydrophobic interactions between analytes and CSP are weakened, and retention decreases (as occasionally does enantioselectivity). The extreme case is when the MP is made up entirely of organic solvent (e.g., acetonitrile or methanol), as in the PO separation mode, which is typically associated with very short retention times. Therefore, the logical approach to improving chiral resolution is to allow for longer retention (as taking place in weaker MP; see example of trimipramine and ketamin in Fig. 2). However, in cases when only partial resolution is achieved with retention times longer than 10 min, further decreasing the organic modifier content in the MP may produce baseline separation only with very long retention times or may not work at all. This latter phenomenon can be explained by an excessive increase in peak widths noticed at long retention times occasionally counterbalancing any benefit in regards of selectivity.

Similarly to achiral RP, methanol exhibits less eluting strength compared to acetonitrile, requiring more methanol than acetonitrile to be present in the MP for similar retention [29]. Based

on this observation, we set the initial MP composition for aqueous (buffer):acetonitrile mixtures to 40:60 (v/v) and for aqueous (buffer):methanol mixtures to 20:80 (v/v) for all RP screening experiments. These initial conditions are expected to insure adequate retention for most test compounds. Whenever enantiomers eluted early (in less than 10 min and only partially resolved) or late (after 20 min with very good resolution), further optimization was attempted by adjusting the eluting strength of the MP. The examples shown in Fig. 3 demonstrate that acetonitrile (in comparison with methanol) may induce a different selectivity of a particular CSP for the same pair of enantiomers and also that one modifier may be more beneficial than the other in achieving acceptable resolution in the shortest time (as in the shortest analysis time and/or with the lowest number of trials). Additionally, it appears that methanol, a protic solvent, in fact does not further disrupt hydrogen bonding interactions between analytes and CSP, an effect that would reduce its utility as organic modifier in RP chiral LC. Such conclusions may be hastily drawn when based on a limited number of test compounds and should be formulated only based on the statistical evaluation of a relevant pool of data (see Section 3.6).

3.3. Effect of mobile phase additives

Basic or acidic MP additives are often required for improving resolution and peak shapes of ionizable analytes in any separation mode [12–14,30]. Aqueous MPs buffered in the pH range 2–5 are
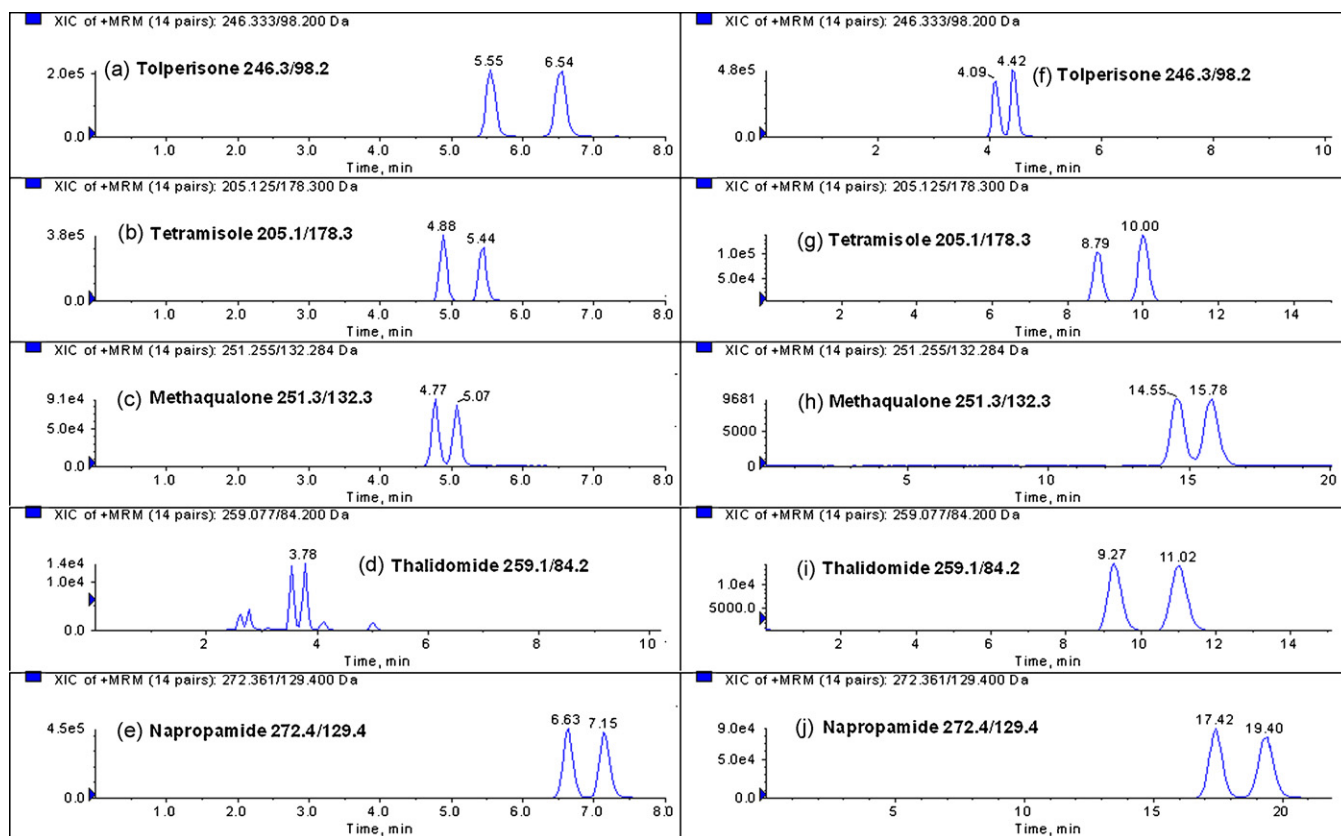
Fig. 3. Effect of choice of mobile phase organic modifier (acetonitrile or methanol) on chiral selectivity. Column – Lux 3 μm Cellulose-2, 150 mm × 2.1 mm i.d.; flow rate – 0.2 ml/min; ESI⁺ MS/MS detection (detector settings as specified in Section 2.2). Analyte names are followed by the specific parent/fragment ion pair monitored in each case. Mobile phase conditions for extracted ion chromatograms on the left – 5 mM ammonium hydrogencarbonate with acetonitrile 4:6 (v/v); extracted ion chromatograms on the right – 5 mM ammonium hydrogencarbonate with methanol 3:7 (v/v).
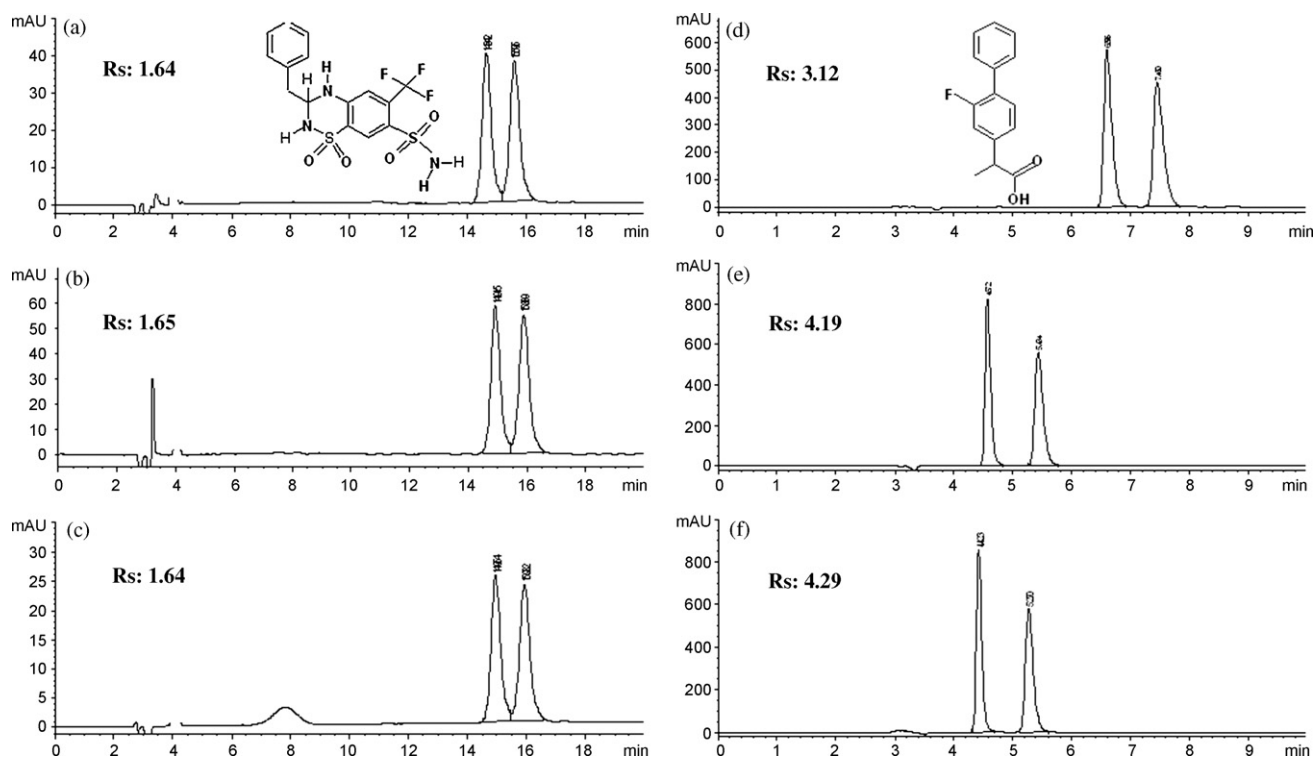
Fig. 4. Effect of choice of acidic additive on the peak shapes and chiral resolution of the acidic racemic compounds bendroflumethiaze (a–c) in mobile phase – 0.1% acetic, formic, or trifluoroacetic acid with acetonitrile 6:4 (v/v) with Lux 5 μm Cellulose-1, 250 mm × 4.6 mm i.d., and flurbiprofen (d–f) in mobile phase – 0.1% acetic, formic, or trifluoroacetic acid with acetonitrile 4:6 (v/v) with Lux 5 μm Amylose-2, 250 mm × 4.6 mm i.d.; flow rate – 1.0 ml/min; UV detection – 220 nm; injection volume – 10 μl; sample concentration – 500 μg/ml.
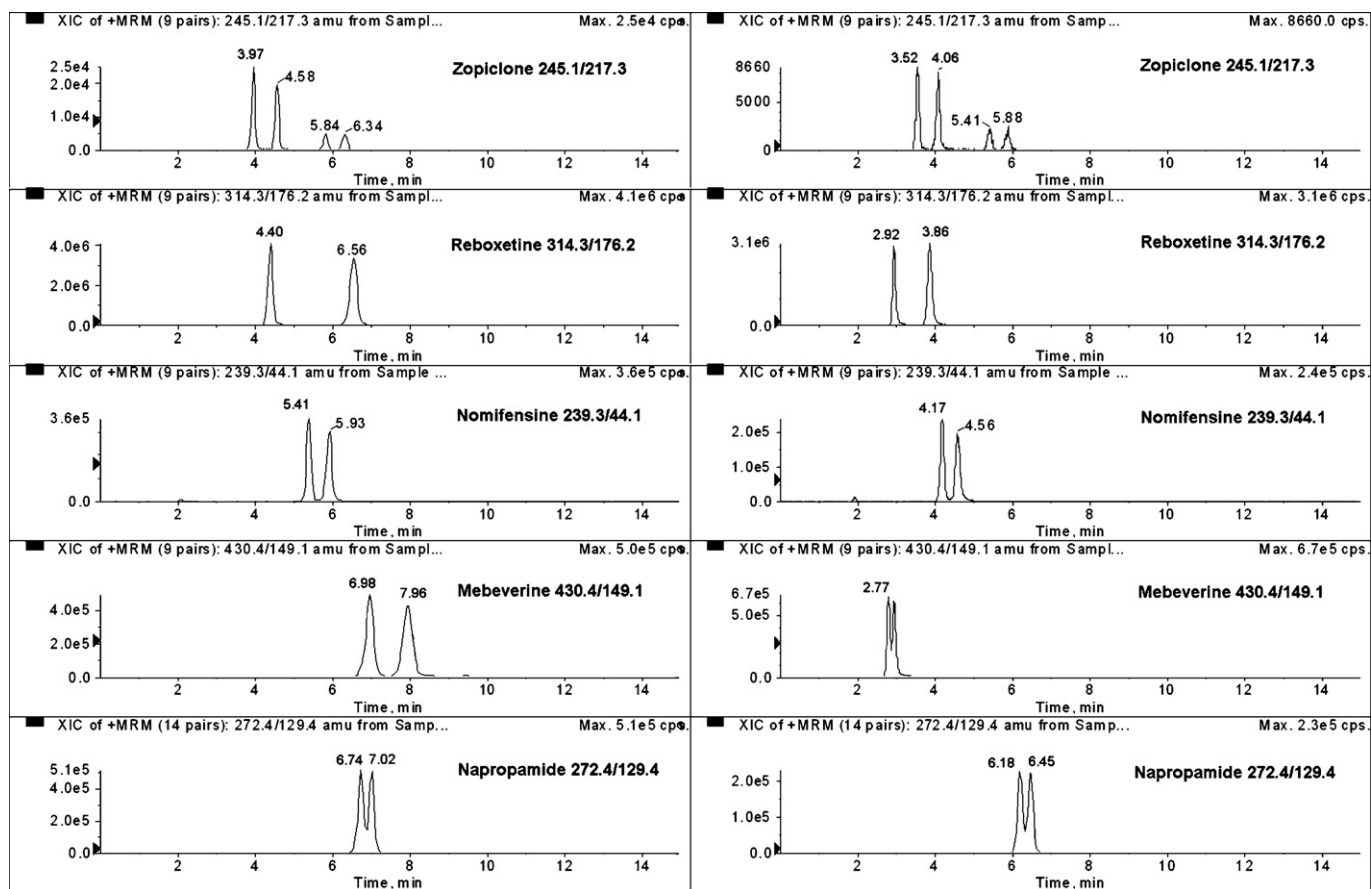
Fig. 5. Chiral resolution in mobile phases containing ammonium hydrogencarbonate (extracted ion chromatograms on the left) or ammonium acetate (extracted ion chromatograms on the right). Column – Lux 3 μm Cellulose-1, 150 mm × 2.1 mm i.d.; flow rate – 0.2 ml/min; MS detector settings as specified in Section 2.2.

effective in the separation of acidic racemates on polysaccharide-based CSPs. The choice of a particular acidic additive is based on the pK_a of the analyte (i.e., the strength of the acidic functionalities present in its structure) and the pK_a of particular additives (trifluoroacetic acid – pK_a 0.59; formic acid – pK_a 3.75; acetic acid – pK_a 4.76). While weakly acidic racemates will respond well to any of the above listed additives achieving similar enantioresolution (see the separation of the weak acid/neutral bendroflumethiazide on Lux Cellulose-1 in Fig. 4a–c), stronger acids resolve better in MP containing a stronger acidic additive (see the separation of flurbiprofen (pK_a 4.33) on Lux Amylose-2 in Fig. 4d–f). Another consideration in choosing an additive relates to the phenomenon termed "memory effect". Trifluoroacetic acid (TFA), while an effective protonating (and ion-pairing) agent, binds intimately to polysaccharide-based CSPs and therefore is hard to remove once a column needs to be converted to (i.e., be used in) a different MP [31]. Therefore, formic and acetic acids are usually preferred over TFA as acidic additives, except when columns can be dedicated to specific additives. In our laboratory formic acid is the first choice of additive in RP MPs for the separation of acidic and neutral racemates. While the latter category of racemates usually does not require the presence of an additive in the RP MP practical considerations (such as minimizing the number of MPs used in routine screening) promote the use of MPs containing additives for all compounds.

Higher pH MPs buffered with ammonium salts such as acetate or hydrogencarbonate (with ammonia) can be effective in the chiral separation of basic (and neutral) racemic compounds [14]. Ammonium salts are also thermally labile, hence fully compatible with MS detectors, and even preparative-applications friendly (as they can be easily removed from the final product). Higher pH mobile phases

are typically considered incompatible with silica-based chromatographic sorbents. The stability of Lux HPLC columns was evaluated in mobile phase containing ammonium hydrogencarbonate and DEA with acetonitrile 5:5 (v/v). No change in column performance was noticed after passing 4000 column volumes of mobile phase pH 8.9. This result demonstrates that these columns can be operated in mobile phase containing aqueous buffer of pH < 9.

The enantioselectivity of Lux CSPs was evaluated in ammonium acetate or ammonium hydrogencarbonate-containing MPs (with acetonitrile as organic modifier) for a large number of racemates. In general, ammonium hydrogencarbonate (pH 7.8 by itself, or pH 8.9 when in combination with 0.1% DEA) provides similar or occasionally better selectivity than ammonium acetate (pH 5.4) as MP aqueous component. As shown in Fig. 5 reboxetine and mebeverine are resolved with better selectivity in mobile phase containing ammonium hydrogencarbonate. To our knowledge, this observation has not yet been reported. In contrast, a recent study by Zhang et al. found that "…enantioselectivity is not influenced very much by the nature of the basic aqueous solutions…" and only that "…comparing to the ammonium acetate solution, the ammonium bicarbonate medium could sometimes afford better peak shapes, therefore higher resolution of the enantiomers." [26]. This discrepancy may be explained by the limited number of test probes investigated in Ref. [26].

The addition of DEA to RP MPs can visibly improve the enantioresolution of some basic compounds (e.g., β-blockers and tricyclic antidepressants; see the examples of nifenolol and trimipramine in Fig. 6). However, it can also severely suppress analyte response in positive mode electrospray (ESI+) MS/MS even when DEA is present in the MP at levels as low as 0.025%. However, DEA
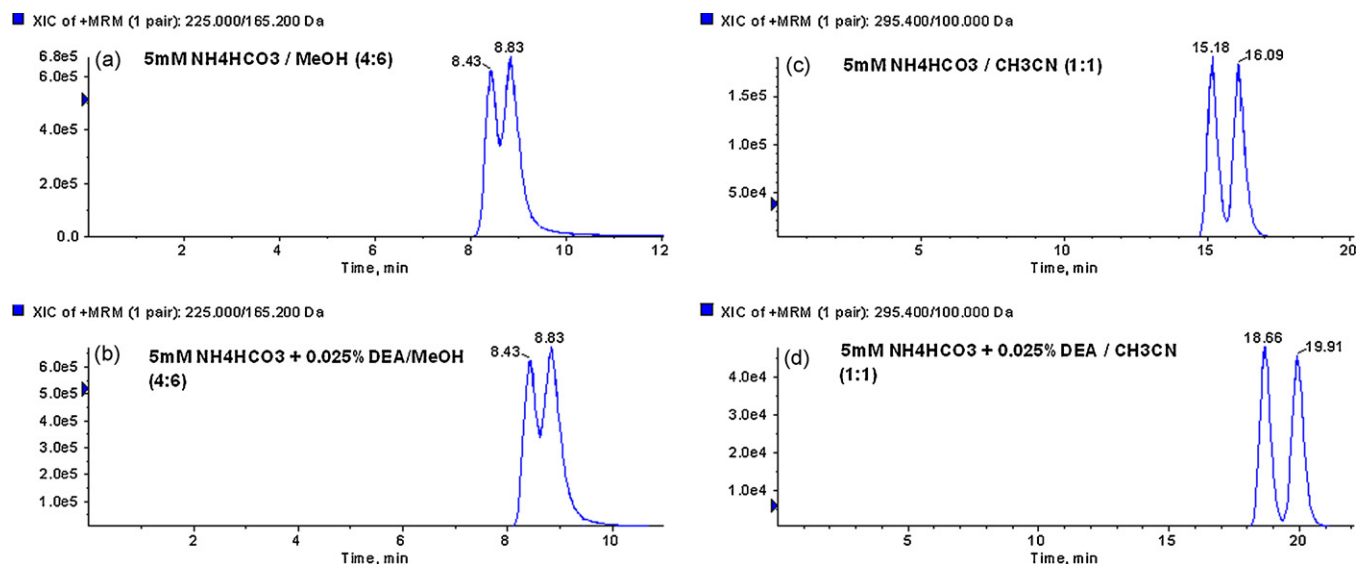
Fig. 6. Effect of DEA as mobile phase additive on the chiral resolution of basic racemates and on analyte response in LC/MS/MS: no DEA in mobile phase (a and c); 0.025% DEA present in the mobile phase (b and d). Column – Lux 3 μm Cellulose-2, 150 mm × 2.1 mm i.d.; flow rate – 0.2 ml/min (detector settings as specified in Section 2.2).

(or an acidic additive) does not affect the enantioresolution of either weakly or moderately basic compounds (e.g., benzodiazepines, imidazoles), or that of neutral racemates. For all these compounds, baseline separation can be achieved in MPs fully compatible with MS detectors (without any DEA or any strong acidic additive such as TFA being present).

3.4. Effect of temperature

Several authors have reported improved resolution on polysaccharide-based CSP when the column was operated at sub-ambient temperature [32–34]. In the current study, the effect of temperature on the resolving power of cellu-

lose tris(3-chloro-4-methylphenylcarbamate) and amylose tris(2-chloro-5-methylphenylcarbamate) CSPs was studied in the range 5–35 °C and compared to that of cellulose tris(3,5-dimethylphenylcarbamate). Test compounds selected for this segment of our study were only partially resolved at room temperature on any of these CSPs. In general, decreasing temperature produces slower mass transfer kinetics, resulting in increased retention and decreased column efficiency (see Fig. 9).

Our results show that the effect of column temperature on chiral resolution varies from case to case, it is unpredictable and not significant on any of the CSPs and the temperature range studied here. As it is known the effect of column temperature on chiral resolution is a function of the balance between enthalpic and enthropic
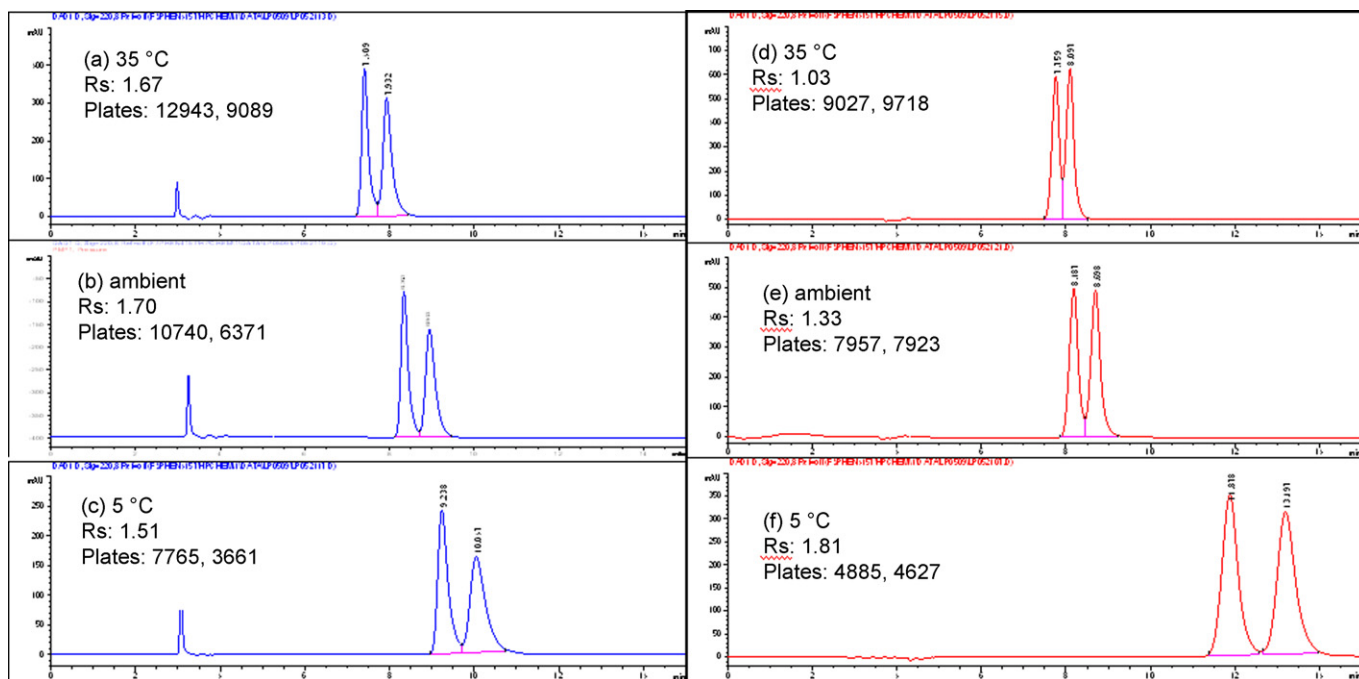

Fig. 7. Effect of column temperature on the chiral resolution of thalidomide (a–c) and fluoxetine (d–f). Mobile phase: 20 mM ammonium hydrogencarbonate with 0.1% DEA and acetonitrile 6:4 (a–c) and 20 mM ammonium hydrogencarbonate with 0.1% DEA and methanol 2:8 (d–f); column – Lux 5 μm Cellulose-1, 250 mm × 4.6 mm i.d; flow rate: 1.0 ml/min; UV detection – 220 nm.

Table 1
Resolution of 16 basic or neutral compounds on Lux Cellulose-1 and Lux Cellulose-2 in RP.

Compounds	Lux 5 μm Cellulose-1	Lux 5 μm Cellulose-2
Benflourex	X	P
Bupropion	X	P
Clenbuterol	X	B
Dichloroisoproternol	X	B
Diltiazem	X	X
Felodipine	X	B
Ketamine	P	B
Labetalol	X	X
Metolazone	B	X
Nicardipine	X	X
Nifenalol	X	B
Nimodipine	X	X
Promethazine	P	X
Sulpride	X	X
Trimipramine	X	B
Verapamil	B	X

B(aseline separation) – Rs > 1.5.
P(artial separation) – 0.80 < Rs < 1.50.
X marginal or no separation - Rs < 0.80.

contributions to the relative retention of enantiomers [34]. Any gains in selectivity achieved by decreasing temperature may be offset by losses in column efficiency (as is the case for thalidomide; see Fig. 7a–c) or in some cases can prevail resulting in improved resolution (as is the case for fluoxetine; see Fig. 7d–f).

3.5. Complementary enantioselectivity in RP to NP and PO modes of separation

In the initial stage of this investigation 16 various basic or neutral compounds for which cellulose tris(3-chloro-4-methylphenylcarbamate) and cellulose tris(3,5-dimethylphenylcarbamate) have shown minimal or no chiral recognition in NP or PO separation modes were screened in RP, in MP composed of aqueous 20 mM ammonium acetate containing 0.1% DEA and acetonitrile. This screening effort led to unexpected results: 11 out of 16 compounds (69%) were at least partially resolved with 8 (50%) baseline resolved (combined results on Lux 5 μm Cellulose-2 and Lux 5 μm Cellulose-1; see Table 1). These results suggest that the RP separation mode may provide a viable solution in cases where other separation modes fail to resolve the pair of enatiomers of interest. The question remains whether RP should be explored as a last resort, only once NP, PO or SFC separation modes have failed providing adequate resolution or could it provide a response sooner in the quest for a CSP/MP combination capable of providing the desired resolution. Furthermore, could preparative applications benefit from (potentially) larger separation factors achieved in RP compared to other separation modes, counterbalancing such considerations as the lower volatility of aqueous-based MPs? Some answers are provided in the next sections.

3.6. Chiral LC screening results in RP

A more extensive study was undertaken with the purpose of evaluating the overall success of RP chiral chromatography in baseline resolving a variety of racemic compounds and comparing it to that of the more commonly explored separation modes NP and PO. The three polysaccharide-based CSPs studied above (including one tris(chloromethylphenylcarbamate) derivative of cellulose and one of amylose) were evaluated in the RP elution mode for the separation of the entire collection of racemates described in Section 3.1. The MP consisted of aqueous ammonium hydrogen-carbonate (with 0.1% DEA) or aqueous 0.1% formic acid mixed with

either 60% acetonitrile or 80% methanol (as starting conditions). Whenever only partial resolution was achieved, further optimization was attempted by following the strategy described in Section 3.2. In practical terms, mobile phase conditions were adjusted to produce separations in less than 20 min with both enantiomers *preferably* eluting in the time interval $10 < t_{R1}$ and $t_{R2} < 20$ min. Nevertheless, we report here cases in which this strategy did not return the desired result: sometimes baseline resolution could be achieved only with excessive retention (e.g., naringenin on Lux Cellulose-1 with acetonitrile as organic modifier; dichloroisoproternol on Lux Cellulose-2 with methanol as organic modifier, or miconazole on Lux Amylose-2 with acetonitrile as organic modifier), and for several compounds resolution could not be improved by increasing retention (e.g., norephedrine on Lux Cellulose-2 and diperodon on Lux Amylose-2; see Tables 2–4).

It is noteworthy mentioning that cypermethrin, a compound identified by Borman et al. as markedly different in regards of its structural descriptors relevant to chirality [12], could not be separated by any of the CSPs investigated here. This result is possibly confirming the unique status of this test probe within the population of chiral test compounds. At the same time, diperodon (placed close to the main cluster in terms of PCA scores) was easily resolved on two of the CSPs, while trans-stilbene oxide was resoled by all three. These observations are significant as they lend themselves to designing a population of test compounds of general use which can meet both the expectations for diversity and for minimal redundancy.

The results tabulated below demonstrate that reversed-phase chiral screening can be very successful, with 120 out of 209 racemic compounds (57%) being baseline resolved in RP on at least one of the Lux CSPs. This result is quite significant considering the fairly large number of chiral compounds included in this study. In less meaningful terms (but in line with the current practice on the subject in the scientific literature) we report an overall success rate of 65% in terms of enantioselectivity (combining both partial and complete resolution) for the RP separation mode.

The traditional cellulose tris(3,5-dimethylphenylcarbamate) CSP (i.e., Lux Cellulose-1) baseline resolved 66 out of the 209 compounds (32%), the novel CSP cellulose tris(3-chloro-4-methylphenylcarbamate) (i.e., Lux Cellulose-2) was able to baseline resolve 59 racemic compounds (28%), while amylose tris(2-chloro-5-methylphenylcarbamate) (i.e., Lux Amylose-2) baseline resolved 63 compounds (30%) (see Tables 2–4; compounds resolved on only one of the three Lux phases in RP are listed in bold letters).

Comparing the success rates of the three separation modes in common use in baseline resolving all racemates included in this study, RP emerges as comparable to or better than NP or PO (see Fig. 8) for every CSP investigated here. This finding demonstrates the (perhaps until now unrecognized) potential of reversed-phase chiral LC of potentially providing a separation solution on the first round of screening on any the three CSPs discussed here, with chances equal to other separation modes. Furthermore, it suggests that screening protocols should be revised to better realize this potential of RP chiral LC. At the same time, it is easy to recognize that the tris(chloromethylphenylcarbamate) derivatives of cellulose and amylose in discussion are CSPs of wide applicability, rivaling such a commonly used CSP like cellulose tris(3,5-dimethylphenylcarbamate). This finding is in line with similar conclusions reached based on a study conducted in the NP and PO separation modes [19] elevating tris(chloromethylphenylcarbamate) derivatives of cellulose and of amylose to the first tier of chiral LC columns to be screened in any laboratory.

The analysis of the data listed in Tables 2–4 reveals further trends in regards to the optimal MP conditions to be adopted on the first round of screening experiments. The initial mobile

Table 2
Retention times and resolution for test compounds baseline resolved on Lux 5 μm Cellulose-1 in RP mode. Buffer composition: Aq1 – 20 mM NH_4HCO_3 + 0.1% DEA; Aq2 – 20 mM NH_4Ac + 0.1% DEA; Aq3 – 20 mM NH_4HCO_3; Aq4 – 0.1% acetic acid.

Compounds	Results			Mobile phase		
	t_{R1}	t_{R2}	Rs	Buffer	% CH_3CN	% CH_3OH
Alprenolol	8.1	9.1	2.1	Aq1	40	–
Althiazide	7.4	8.2	2.3	Aq1	40	
Aminoglutethimide	5.4	5.9	3.4	Aq1	60	–
Ambucetamide	8.0	13.1	14.9	Aq1	60	–
Atropine	8.0	8.7	1.5	Aq3	–	60
Bendzoflumethiazide	14.1	15.2	1.6	Aq4	40	–
	4.3	4.8	2.2	Aq4	–	80
Benzoin	6.7	7.6	4.3	Aq1	60	–
Betaxolol	15.2	16.9	1.6	Aq1	30	–
Bifonazole	21.0	22.6	1.7	Aq1	60	–
Bopindolol	7.2	8.3	2.7	Aq1	–	90
Bupivacaine	13.4	13.6	1.5	Aq1	–	70
Bupranolol	14.6	16.9	2.5	Aq1	–	60
Butaclamol	3.2	8.6	8.1	Aq1	80	–
Carbinoxamine	21.0	22.6	1.8	Aq1	30	–
Carprofen	7.7	8.7	3.1	Aq1	60	–
1-(2-Chlorophenyl)-1-(4-chlorophenyl)-2,2-dichloroethane	8.6	9.6	2.5	Aq1	–	90
Citalopram	8.0	8.6	2.0	Aq1	60	–
1,1-Dihydroxy-6,6-dimethylbipheyl	5.3	5.9	2.7	Aq1	–	80
(I)-N-(3,5-Dinltrobenzyl)-α-methylbenzylamine	30.9	32.8	1.5	Aq1	50	–
Econazole	8.8	9.6	1.8	Aq1	–	80
Etodolac (a)	8.7	9.5	2.6	Aq4	60	–
Enilconazole	11.3	12.1	1.6	Aq1	–	80
Etozolin	8.3	8.6	1.9	Aq1	60	–
Flavanone	12.2	12.9	1.8	Aq3	60	
	8.9	10.8	5.2	Aq3	–	90
Fluoxetine (at 5°C)	11.9	13.2	1.8	Aq1	–	80
Halofantrine	13.8	16.0	2.9	Aq1	–	90
Homatropine	5.6	6.8	4.4	Aq3	–	80
5-(p-hydroxyphenyl)-5-phenylhydantoin	3.9	4.8	5.2	Aq1	60	–
Hydroxyzine	9.9	11.1	2.2	Aq1	–	80
Indapamide	5.5	7.1	4.7	Aq1	60	–
Isoamarine	11.4	20.8	9.5	Aq1	–	80
Isoxsuprine	20.4	22.5	1.5	Aq3	–	60
Lorazepam	4.9	7.7	10.2	Aq1	60	–
Methoxamine	6.4	7.1	2.0	Aq1	–	50
Metofoline	11.6	12.4	1.8	Aq1	60	–
Mebeverine	13.7	15.2	2.8	Aq1	60	–
Meclizine	10.2	11.0	1.7	Aq3	80	–
Mephenesine	7.2	7.9	2.6	Aq1	30	–
Mephenytoin	5.1	5.5	2.1	Aq1	50	–
5-Methyl-5-phenyl-hydantoin	5.0	5.4	2.0	Aq3	30	–
Methocarbamol	4.9	5.7	3.9	Aq1	–	80
Metolazone	9.6	10.4	1.7	Aq1	40	–
Metoprolol	14.7	16.3	1.8	Aq1	20	–
	19.8	21.8	1.6	Aq1	–	50
Mianserine	8.2	9.6	2.8	Aq1	–	90
Miconazol	44.0	50.0	1.7	Aq1	–	80
Molindone	11.8	12.8	2.0	Aq1	30	–
	6.3	6.7	1.6	Aq1	–	80
Naringenin	29.8	31.7	1.6	Aq4	30	–
Napropamide	10.2	11.5	2.3	Aq1	–	80
Nefopam	8.8	9.4	1.5	Aq1	50	–
Nisoxetine	4.2	5.2	4.2	Aq1	60	–
Nomifensine	7.4	7.9	2.2	Aq1	60	–
Orphenadrine	7.2	9.6	7.7	Aq1	60	–
Oxazepam	5.9	10.5	11.7	Aq1	60	–
Oxprenolol	16.1	20.8	3.4	Aq1	60	–
Phenoxybenzamine	8.0	10.1	5.5	Aq1	–	90
Pindolol	5.8	8.6	5.9	Aq1	–	80
Proglumide (a)	6.9	7.9	3.3	Aq1	–	80
Reboxetine	4.3	5.7	6.2	Aq1	80	–
Sulconazole	11.7	12.5	1.5	Aq1	–	90
Temazepam	6.1	7.2	4.6	Aq1	60	–
Terfenadine	19.8	21.6	1.5	Aq1	60	–
Thalidomide	8.4	9.1	1.7	Aq1	40	–
Toliprolol	4.6	6.0	4.5	Aq1	50	–
Tramadol	5.9	6.3	2.0	Aq1	50	–
Trichlormethiazide	3.2	4.1	3.6	Aq3	–	60
trans-Stilbene oxide	17.7	19.4	2.8	Aq1	60	–
	8.5	11.1	4.6	Aq1	–	90

Table 2 (*Continued*)

Compounds	Results			Mobile phase		
	t_{R1}	t_{R2}	Rs	Buffer	% CH$_3$CN	% CH$_3$OH
Verapamil	26.8	33.2	1.5	Aq1	35	–
Warfarin (a)	7.1	9.3	7.0	Aq4	60	–
Zopiclone	11.4	15.0	4.7	Aq1	–	90

NH$_4$Ac – ammonium acetate. DEA – diethylamine.

Table 3
Retention times and resolution for test compounds baseline resolved on Lux 5 μm Cellulose-2 in RP mode. *Buffer composition*: Aq1 – 20 mM NH$_4$HCO$_3$ + 0.1% DEA; Aq2 – 20 mM NH$_4$Ac + 0.1% DEA; Aq3 – 20 mM NH$_4$HCO$_3$; Aq4 – 0.1% acetic acid.

Compound	Results			Mobile phase		
	t_{R1}	t_{R2}	Rs	Buffer	% CH$_3$CN	% CH$_3$OH
Adrenaline	5.6	6.7	2.8	Aq3	–	60
Althiazide	4.2	4.6	2.6	Aq1	50	–
Ambucetamide	7.4	17.8	12.7	Aq1	–	90
Aminoglutethimide	5.5	6.0	2.2	Aq1	60	–
Bendroflumethiaze	8.5	10.1	3.3	Aq4	40	–
Benzoin	17.6	19.3	1.7	Aq1	–	60
Bifonazole	8.5	9.6	2.1	Aq1	–	90
Bromopheniramine	2.8	7.9	17.8	Aq1	–	80
Butaclamol	9.6	14.6	7.5	Aq1	70	–
β-(Sec-butyl)-phenethyl alcohol	5.84, 6.2	6.7	1.8, 1.5	Aq1	50	–
Chlorpheniramine	2.6	7.0	22.7	Aq1	60	–
Cisapride	11.2	14.7	5.0	Aq1	60	–
Clenbuterol	11.3	12.3	1.8	Aq1	30	–
Dichloroisoproternol	36.8	46.8	1.6	Aq3	–	50
Diperodon	8.5	9.7	2.8	Aq1	60	–
Econazole	16.5	19.6	3.1	Aq1	70	–
Enilconazole	13.6	15.4	2.4	Aq1	60	–
Eserine	5.7	10.2	10.4	Aq1	–	80
Ethodin (a)	9.3	10.5	1.9	Aq1	–	50
Etodolac (a)	8.2	8.9	1.7	Aq4	50	–
Etozolin	10.7	15.8	8.9	Aq1	60	–
Hexobarbital	5.6	6.0	2.3	Aq1	60	–
	7.6	9.8	4.8	Aq1	–	80
Isoamarine	14.3	15.6	2.2	Aq1	50	–
Isoproterenol	5.8	7.9	5.0	Aq1	–	80
Isoxsuprine	4.2	4.7	2.8	Aq3	60	–
Ketamine	32.9	35.1	1.5	Aq1	30	–
Ketorolac (a)	8.3	9.1	1.5	Aq4	60	–
Lorazepam	6.9	7.8	1.7	Aq3	–	80
	7.1	8.0	1.8	Aq3	–	80
Lumefantrine	52.0	60.0	2.2	Aq1	–	80
Mephenytoin	10.1	11.3	1.9	Aq1	–	60
Methaqualone	6.2	1.6	6.6	Aq1	60	–
Methyl-p-tolyl sulfoxide	7.8	8.4	1.6	Aq1	–	80
Metofoline	12.3	13.3	1.9	Aq1	60	–
Miconazole	14.3	17.0	3.8	Aq1	80	–
Midodrine	4.2	4.8	2.3	Aq1	–	80
Milnacipran	11.5	13.9	2.4	Aq1	–	60
Napropamide	15.5	16.9	2.2	Aq1	50	–
Nifenalol	9.1	9.9	1.7	Aq1	–	60
Nisoldipine	7.5	8.3	2.2	Aq3	60	–
	7.6	8.4	1.7	Aq1	60	–
Nomifensine	6.5	7.6	4.2	Aq1	60	–
Norephedrine	4.4	4.8	1.6	Aq1	–	60
Omeprazole	6.7	9.1	5.3	Aq1	60	–
Pheniramine	3.3	6.8	17.8	Aq1	60	–
Pindolol	8.0	8.7	1.8	Aq1	30	–
Praziquantel	11.3	16.4	5.9	Aq1	80	–
Proglumide (a)	11.7	13.1	1.6	Aq4	–	70
Reboxetine	6.6	7.3	1.8	Aq1	60	–
Terfenadine	16.1	19.8	4.0	Aq1	–	80
Tesicam	6.8	7.7	2.5	Aq1	50	–
Tetramisole	6.6	7.4	2.9	Aq1	60	–
Thalidomide	5.3	6.4	4.9	Aq1	60	–
Tolperisone	10.0	11.6	3.7	Aq1	60	–
trans-Stilbene oxide	8.5	11.2	7.2	Aq3	60	–
	8.7	11.9	7.1	Aq3	–	80
Trichlormethiazide	3.4	3.9	1.9	Aq3	30	–
Trimipramine	23.0	24.6	1.7	Aq1	50	–
Tropicamide	19.7	22.4	1.7	Aq1	–	60
Warfarin (a)	7.0	7.6	1.6	Aq4	60	–
Zopiclone	10.4	14.8	6.6	Aq1	61	–

Table 4

Retention times and resolution for test compounds baseline resolved on Lux 5 μm Amylose-2 in RP mode. Buffer composition: Aq1 – 20 mM NH_4HCO_3 + 0.1% DEA; Aq2 – 20 mM NH_4Ac + 0.1% DEA; Aq3 – 20 mM NH_4HCO_3; Aq4 – 0.1% acetic acid.

Compounds	Results			Mobile phase		
	t_{R1}	t_{R2}	Rs	Buffer	% CH_3CN	% CH_3OH
Alprenolol	6.3	6.9	2.1	Aq1	30	–
Ambucetamide	7.8	10.9	5.1	Aq1	60	–
Aminoglutethimide	5.6	6.5	2.1	Aq1	60	–
Benzoin	11.6	12.9	2.7	Aq1	40	–
	6.6	7.5	1.6	Aq3	–	80
Betaxolol	4.8	6.0	3.8	Aq1	60	–
Bisoprolol	5.7	6.5	2.3	Aq1	40	–
Bopindolol	5.7	7.4	4.2	Aq1	60	–
Bromopheniramine	6.0	10.1	6.8	Aq1	60	–
Bupranolol	11.4	12.5	1.9	Aq1	30	–
Butaclamol	10.0	13.2	4.4	Aq1	60	–
β-(Sec-butyl)-phenethyl alcohol	11.2	12.2	2.3	Aq1	80	–
Carazolol	10.9	12.1	1.6	Aq1	30	–
Chlorpheniramine	5.4	8.5	7.8	Aq1	60	–
1,1-Dihydroxy-6,6-dimethylbipheyl	10.7	11.9	2.5	Aq1	40	–
Dimethindene	10.0	10.9	1.7	Aq1	40	–
Diperodon	6.3	7.3	1.5	Aq1	60	–
Econazole	15.0	16.8	2.4	Aq1	60	–
Enilconazole	11.0	11.8	1.7	Aq1	50	–
Etozolin	7.1	10.3	6.5	Aq1	60	–
Flavanone	5.3	6.7	6.9	Aq1	80	–
	7.5	11.3	4.7	Aq1	–	90
Flurbiprofen (a)	7.6	8.5	3.5	Aq4	60	–
Halofantrine	7.4	14.2	4.8	Aq1	60	–
Hexobarbital	5.3	5.9	2.2	Aq1	40	–
Ifenprodil	4.9	7.0	4.2	Aq1	60	–
Isoamarine	6.8	17.0	8.2	Aq1	60	–
Kavain	6.9	7.5	2.6	Aq1	50	–
Ketamine	5.3	5.7	2.1	Aq1	60	–
Ketorolac (a)	12.2	14.1	4.1	Aq4	60	–
Lansoprazole	3.7	4.4	2.7	Aq1	60	–
Laudanosine	3.0	5.4	6.3	Aq1	–	70
Linalool	10.5	11.3	1.7	Aq1	60	–
Meclizine	9.7	11.4	2.2	Aq3	80	–
Mephenesin	7.5	8.0	1.6	Aq1	20	–
Mesoridazine	7.9	8.4	1.6	Aq1	30	–
Methaqualone	5.3	6.0	2.8	Aq1	60	–
5-Methyl-5-phenyl-hydantoin	4.0	4.4	1.9	Aq1	30	–
Methoxamine	4.0	4.5	1.5	Aq1	30	–
Metofoline	9.6	12.1	5.0	Aq1	60	–
Metomidate	4.9	6.0	4.9	Aq1	60	–
Metoprolol	4.9	5.6	2.2	Aq1	40	–
Miconazole	20.7	21.9	1.7	Aq1	60	–
Nefopam	5.0	6.2	4.8	Aq1	60	–
Nomifensine	4.9	5.7	3.5	Aq1	60	–
Norephedrine	3.9	4.3	1.6	Aq1	30	–
Norketamine	4.8	5.2	2.2	Aq1	60	–
Omeprazole	8.9	11.1	1.9	Aq1	30	–
Ornidazole	3.6	4.6	6.1	Aq1	60	–
Oxazepam	5.8	6.8	2.5	Aq1	60	–
Oxybutynin	7.3	7.9	2.0	Aq1	60	–
Pheniramine	4.3	5.3	4.5	Aq1	60	–
Praziquantel	8.2	11.2	3.6	Aq1	60	–
Proglumide (a)	5.7	6.5	2.2	Aq4	60	–
Propafenone	5.7	13.1	7.4	Aq1	80	–
Propiomazine	12.0	13.1	1.6	Aq3	60	–
Propranolol	6.7	7.3	2.2	Aq1	40	–
Sotalol	3.6	4.0	1.6	Aq1	30	–
Sulfinpyrazone (a)	18.3	19.7	1.8	Aq4	60	–
Suprofen (a)	7.4	8.3	3.2	Aq4	60	–
Temazepam	5.1	12.5	14.3	Aq1	80	–
Tesicam	4.5	5.6	4.2	Aq1	60	–
Tetramisole	6.2	6.7	2.3	Aq1	60	–
Thalidomide	10.6	11.6	1.6	Aq1	30	–
trans-Stilbene oxide	12.0	15.2	6.6	Aq1	60	–
	8.3	10.8	4.7	Aa1	–	90
Warfarin (a)	7.5	8.3	1.6	Aq4	60	–

phase composition of 60% acetonitrile or 80% methanol seems justified by the large number of successful separations achieved at these concentrations of organic modifiers. While a significant subset of the baseline resolved racemic compounds required further

MP optimization in order to improve resolution (namely 28 out of 118; 24%), this subset included mostly poorly retained compounds in buffer/acetonitrile MP, resolving with Rs < 2, or eluting with partial resolution in spite of longer retention. This phenomenon
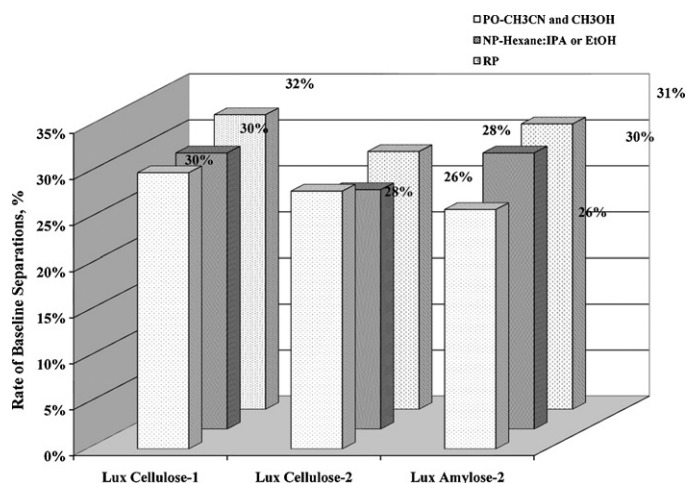
Fig. 8. Success rates of cellulose tris(3-chloro-4-methylphenylcarbamate), amylose tris(2-chloro-5-methylphenylcarbamate) and cellulose tris(3,5-dimethylphenylcarbamate) CSPs in baseline resolving racemic compounds in three commonly used separation modes.

was less often observed in methanol containing MP with less than 8% of baseline resolved racemic compounds requiring less than 70% methanol to be resolved. This last observation leads to another comparison: statistically more enantioseparations were achieved in RP with acetonitrile-containing MP than with methanol on Lux CSPs, suggesting that hydrogen bonding between analyte molecules and CSPs may in fact be disrupted by methanol. Lux Cellulose-1 resolved 28 compounds in methanol compared to 41 in acetonitrile, while Lux Cellulose-2 resolved 21 compared to 37. Moreover, the chiral recognition ability of Lux Amylose-2 seems to be dramatically impaired by methanol, with only 3 compounds resolved, compared to 61 in acetonitrile. This finding cannot be explained solely by the competitiveness of methanol in forming hydrogen bonds with the amylose-based CSP, as such bonds form with cellulose-based CSPs as well. A possible explanation resides in the particular conformation amylose tris(2-chloro-5-methylphenylcarbamate) may adopt compared to the other CSPs.

3.7. The potential of RP chromatography for the purification of chiral compounds

Chiral separations are developed both for analytical and purification purposes. The current practice of screening CSPs primarily in NP, SFC and PO separation modes when the ultimate goal is HPLC purification may be misguided. Commonly invoked reasons for avoiding RP purification are faster solvent removal from useful fractions containing only volatile solvents and potential decomposition of target compounds during a longer solvent evaporation process. These concerns may be justified in particular cases but may be secondary or of no concern at all in many others. After all, the most widely used separation mode in HPLC purification is RP. For example, the largest scale purification processes in the world (namely insulin and taxol purifications) are conducted in RP [35,36]. Similarly, combinatorial libraries of small MW compounds generated in pharma and most synthetic peptides (small and large) are purified in RP [37,38]. This paper draws attention to the possibly unjustified current practice of conducting the purification of chiral species primarily in other separation modes than RP.

Preparative applications typically require high resolution between target compound(s) and closely eluting impurities at analytical loads. The chromatographic parameter closely monitored when developing preparative applications is selectivity. With chiral separations this parameter can be misleading, as occasionally enantiomer peaks can be extremely wide, counterbalancing the benefits of a large selectivity factor. Therefore, resolution values may be a more direct measure of the preparative potential of a particular chiral separation. Baseline resolution of $Rs < 2$ is hardly conducive to high load (i.e., high productivity) in preparative chromatography. Therefore, it is of interest to examine the distribution of resolution values achieved in NP and RP separation modes on the Lux CSPs for the population of test compounds examined here (normal phase data was retrieved from a previous study [39]). For this comparison, resolution values were grouped in four categories: $Rs < 2$; $2 < Rs < 3$; $3 < Rs < 4$ and $Rs > 4$. A histogram of Rs values for each separation mode and CSP is shown in Fig. 9. The data show a fairly scattered distribution of Rs values across columns and separation modes. The values of most interest to preparative chromatographers ($Rs > 3$) seem to be achieved with similar frequencies on all columns and
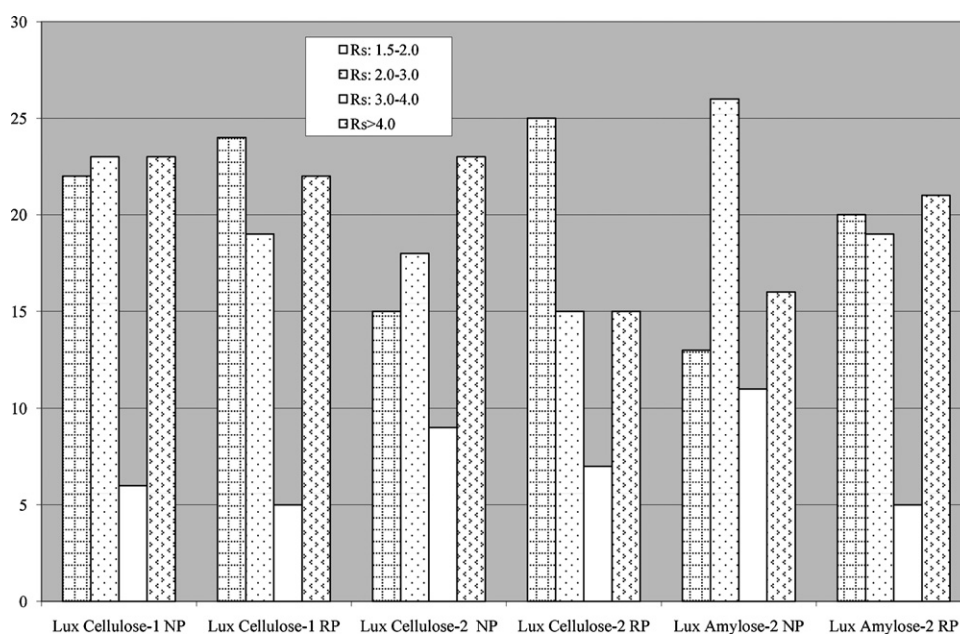

Fig. 9. Histogram of resolution values on three Lux CSPs in NP and RP separation modes.

separation modes, with a small advantage for Lux Cellulose-1 and NP. In our opinion, all separation modes should be explored for the purification task at hand, with the mode providing the largest loading at the desired purity and acceptable recovery being preferred over the other. Shorter solvent removal times may be secondary with lengthy purification processes based on separations providing only low specific loading, while target decomposition during fraction work-up is not a general concern but only compound specific.

3.8. Chiral LC applications with MS detection

RP eluents are compatible with MS detection as long as they contain only volatile additives (as discussed earlier in Section 3.3) as well as with aqueous samples of biological origin such as serum, plasma and urine (in which case no sample blow-down and reconstitution in a compatible solvent are required). Conversely, normal phase eluents used extensively in chiral LC (namely mixtures of alkanes and low MW alcohols) are generally considered incompatible with atmospheric pressure ionization mass spectrometry (API MS) due to possible explosion hazard when such flammable solvents as hexanes are introduced at high flow rate into a heated API source interfacing the HPLC with the MS detector [1]. Nevertheless, several workers have developed NP LC/MS assays [40–44]. In order to minimize any hazard, post-column addition of a large portion of MS-compatible solvent prior to introduction into the heated ion source or alternatively, decreasing the temperature of the heated nebulizer have been explored as possible solutions [40–43]. However, both approaches result in some loss in sensitivity due to the dilution of analyte bands emerging from the HPLC column or to the incomplete desolvation of analyte molecules adversely affecting ionization efficiency, respectively. In the current study we explore

the use of mobile phases fully compatible with MS detection, hence keeping method sensitivity unaffected.

Chiral separations are typically first attempted on long columns (250 mm length) allowing for longer interaction between enantiomers and the CSP in the hope of achieving a more effective discrimination between the two species to be separated. For all LC/MS applications we chose columns in the 150 mm × 2.1 mm i.d. dimensions packed with 3 μm particles, seeking to maintain the highest resolving power with the reduced column length and diameter, shorten analysis times and accommodate API sources typically tolerating lower flow rates. The major challenge under such conditions was to attain sufficient resolution. Needless to say, in *favorable* situations the column length can be further reduced upon method optimization for even shorter analysis times.

It is worthwhile to remember that *most* of the separations reported in Tables 2–4 were generated in MPs not compatible with MS detection (containing DEA as additive). Still, many of them can be reproduced with adequate resolution without adding DEA in the MP as demonstrated earlier (Fig. 5). Further examples shown in Fig. 10 clearly demonstrate that in spite of some compromises in regards to peak shapes (especially with strongly basic compounds, as mentioned before) and somewhat lower chromatographic efficiency associated with narrow bore columns, many compounds can be successfully resolved under conditions typically used in LC/MS applications. Each extracted ion chromatogram shown in Fig. 10 is annotated with the specific MP conditions conducive to the separation demonstrated.

While LC/MS analysis using low-pH MP is common practice, the same cannot be said about analysis conducted at high pH in spite of the obvious benefits in terms of chromatographic performance [45]. The one significant exception is the current practice (part of
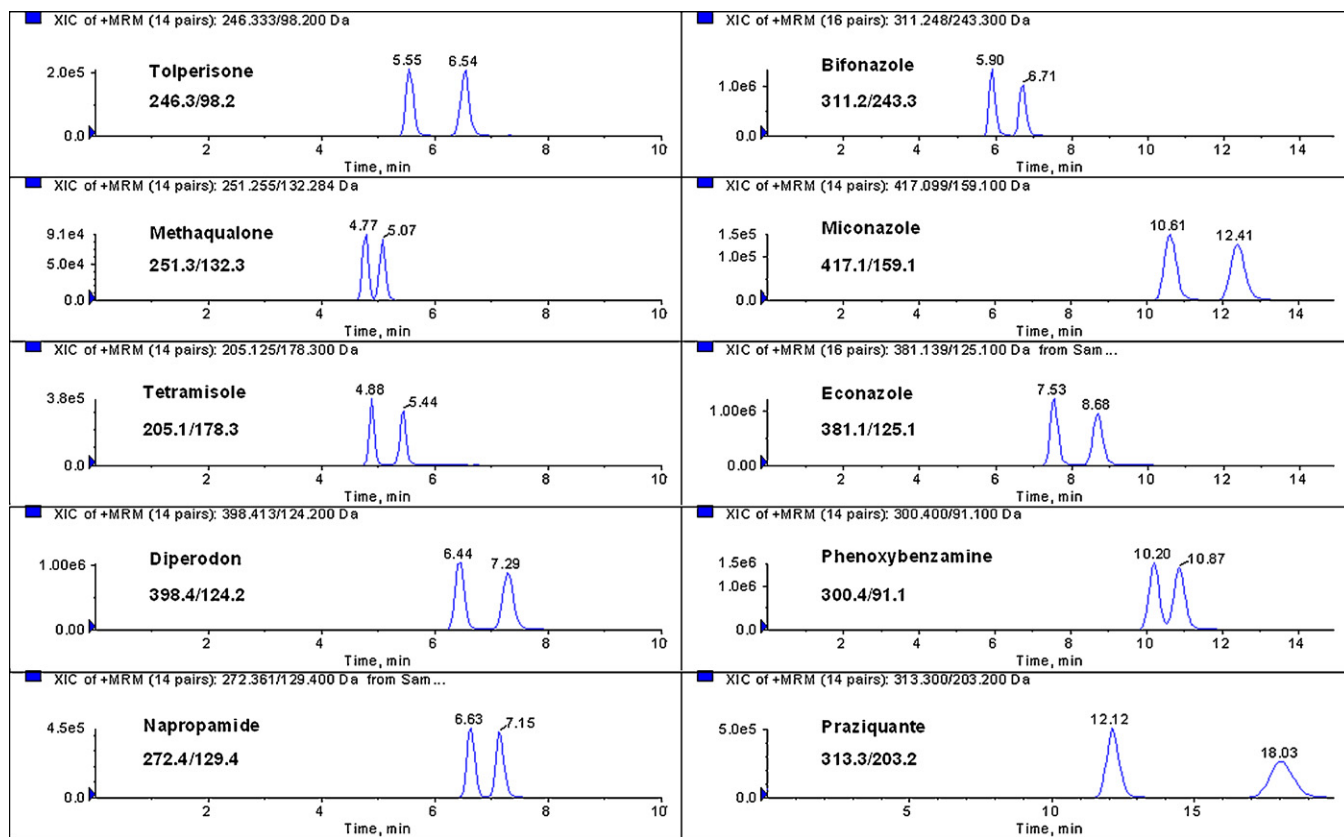

Fig. 10. Examples of successful chiral separations on Lux 3 μm Cellulose-2 150 mm × 2.1 mm i.d. in mobile phase containing no DEA as additive. Mobile phase: 5 mM ammonium hydrogencarbonate with acetonitrile 4:6 (extracted ion chromatograms on the left) and 5 mM ammonium hydrogencarbonate with methanol 1:9 (extracted ion chromatograms on the right); flow rate 0.2 ml/min; MS detector settings as specified in Section 2.2.

drug discovery) of analyzing some combinatorial libraries in high pH MP with the view of extending positive results to the small scale purification of the same compounds. Any concerns regarding the negative impact of high pH MPs on analyte response in ESI⁺ MS were fully addressed in several recent studies on the topic [45–47]. This work demonstrated that analyte response is comparable in high pH MPs to low pH (such as 0.1% formic acid containing MP) or better. Therefore, the use of mobile phases containing ammonium hydrogen carbonate solutions in chiral LC/MS analysis should be beneficial both in regards of enantioselectivity and MS sensitivity.

4. Conclusions

RP chiral LC is complementary to NP and PO separation modes for the successful resolution of racemic compounds on both cellulose tris(3,5-dimethylphenylcarbamate)-based CSP and the newer amylose or cellulose tris(chloromethylphenylcarbamate)s such as cellulose tris(3-chloro-4-methylphenylcarbamate) and amylose tris(2-chloro-5-methylphenylcarbamate) CSPs. Furthermore, RP shows equal or better potential for successful chiral LC and LC/MS analysis when compared to other separation modes.

Diethylamine (as additive present in the MP in addition to the buffer salt) can improve the chiral resolution of strongly basic compounds, but it has a negative effect on analyte response in ESI⁺ MS(/MS) detection even at very low concentration levels (e.g., 0.025%). Decreasing the organic modifier (acetonitrile or methanol) content in the RP MP has the expected effect of increasing retention and often of improving enantioselectivity; therefore, adjusting the eluting strength of the MP is essential to optimizing chiral resolution. The column temperature has only a limited effect on chiral resolution and this effect is compound dependent; its optimization (part of method development) may be useful in cases when only limited resolution is achieved with a combination of CSP and MP particularly favored out of other considerations.

Typical MPs used in RP chiral LC/MS for the separation of basic and neutral racemates are made of aqueous ammonium hydrogencarbonate or acetate as buffer and acetonitrile or methanol as organic modifier. Ammonium hydrogencarbonate is the preferred buffer salt for chiral LC with ESI⁺ MS detection for the successful separation and detection of most of the basic pharmaceutical racemic compounds. Ammonium acetate is a viable alternative to ammonium hydrogencarbonate; it may be less successful in providing baseline resolution. Aqueous formic acid with acetonitrile or methanol can be successfully used in the separation of acidic and neutral racemates.

Cellulose tris(3-chloro-4-methylphenylcarbamate) and amylose tris(2-chloro-5-methylphenylcarbamate) emerge as CSPs of wide applicability in all commonly used separation modes rivaling the well established CSPs based on tris(dimethylphenyl-carbamate) derivatives of cellulose and amylose. Screening protocols may be dramatically improved by including tris(chloromethylphenylcarbamate) derivatives of cellulose and amylose in the set of chiral columns preferentially screened in any laboratory.

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