Journal of Chromatography A, 1234 (2012) 72–79

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Combined use of isopropylamine and trifluoroacetic acid in methanol-containing mobile phases for chiral supercritical fluid chromatography

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ARTICLE INFO

Article history:
Available online 19 November 2011

Keywords:
Isopropylamine
Trifluoroacetic acid
Mobile phase additives
SFC
Methanol-containing mobile phase
Chiral separations

ABSTRACT

In chiral supercritical fluid chromatography (SFC), mobile-phase additives are often used to improve enantioseparations and peak shapes. An acidic or basic additive is chosen, depending on the nature of the compound. This work highlights the simultaneous use of the acidic additive trifluoroacetic acid (TFA) and the basic additive isopropylamine (IPA) in supercritical fluid chromatography for enantioseparations. To evaluate the combination of TFA and IPA, 59 chiral pharmaceutical compounds were analyzed on four polysaccharide-based chiral stationary phases (CSPs): Lux® Cellulose-1, Lux® Cellulose-2, Lux® Cellulose-4 and Lux® Amylose-2. The results show that an important increase in enantioselectivity of the chromatographic system can occur when combining trifluoroacetic acid and isopropylamine in the mobile phase (MP), compared to the individual use of these additives. However, the combination of isopropylamine and trifluoroacetic acid in a supercritical methanol-containing mobile phase can also lead to problems as a result of the formation of salt complexes between the two additives. Combining the additives trifluoroacetic acid and isopropylamine and taking the appropriate measures to avoid salt formation, *i.e.* reducing the additives' concentrations, can lead to simpler chiral SFC screening conditions that display even broader enantioselectivity.

1. Introduction

Chromatographic techniques using chiral stationary phases have proven to be the most successful means to achieve chiral separations [1,2]. In an attempt to enable faster chiral method development, generic chiral screening strategies are defined. These screenings consist of a limited number of experiments and aim to give a quick idea about the enantioselectivity of certain chromatographic systems towards a racemate [3]. As a consequence, three important requisites are inherent to a screening step: it (i) should be fast, (ii) needs to display a broad applicability and (iii) broad enantioselectivity, in order to separate most of the racemates with at least one of the screened systems. When considering the different CSPs, a dominant position among the current commercially available is taken by the polysaccharide-based, since they display a broad enantioselectivity and an easy accessibility [4]. High-pressure liquid chromatography (HPLC) utilizing polysaccharide CSPs remains the most widely used technique for enantioseparations in the pharmaceutical industry [5,6]. However, some drawbacks are related to this technique, such as the potentially relatively long analysis times that may limit the overall throughput and the high consumption of toxic and flammable solvents. In addition, upscaling of the chiral separation method to a preparative level is not always feasible in each mode, especially in reversed-phase liquid chromatography. These issues stimulated the search for more efficient and environmentally friendly alternatives.

In this context, supercritical fluid chromatography has regained interest over the past years as a valuable alternative chromatographic technique for chiral separations [7,8]. As in HPLC, polysaccharide-based stationary phases are also most successful in chiral SFC [9]. SFC uses a supercritical mobile phase, mostly carbon dioxide- (CO_2) based, which results in higher diffusivity and lower viscosity than liquid chromatography mobile phases. Therefore, SFC offers the benefit of higher flow rates compared to conventional HPLC, thus reducing column-equilibration- and analysis times, and enabling a higher throughput. In addition, SFC methods have a lower consumption of organic solvents (methanol is usually added as organic modifier to the mobile phase, see further) and can therefore be considered environmentally friendly and less expensive [7,9–13]. As a result, SFC has the potential to replace HPLC as first choice technique for enantioseparations and purifications in drug discovery and development processes [12–15].

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doi:10.1016/j.chroma.2011.11.023

Taking into account the non-polar nature of CO_2, which has a polarity comparable to that of hexane, SFC can be considered as a normal-phase technique [16]. This implies that the analysis of polar compounds requires the use of polar organic modifiers in the CO_2-based mobile phase to adjust the elution strength [11,12,17]. Most commonly used modifiers are methanol (MeOH), ethanol, isopropanol and acetonitrile. They improve the chromatographic results mainly through an increase in mobile phase polarity and density, leading to an increased solvent strength [18].

However, the addition of a polar organic modifier to the mobile phase may not be sufficient to yield acceptable chromatographic results [11,17]. Very polar or basic compounds, such as amines, may fail to elute or may elute with distorted peak shapes because of their strong interaction with the silica of the stationary phase. For these reasons an additive, such as isopropylamine or trifluoroacetic acid, is often added to the mobile phase, usually at concentrations of 0.1–2.0% [17,19]. Depending on the nature of these additives, different interactions and effects occur which are to date not fully elucidated. Basic additives (amine-derivatives) mask active silanol sites on the stationary phase, hence decreasing the non-specific retention of basic analytes and increasing the enantioselectivity. In addition, these amines are believed to compete with basic analytes for binding on specific sites of the stationary phase, resulting in mixed effects on the enantioselectivity [19]. Finally, the ionization of the analytes plays an important role, especially when the interactions leading to the separation mainly have a non-ionic nature, as is the case for the neutral polysaccharide-based CSPs [19]. An essential function of basic as well as acidic additives is to suppress the ionization of strong basic and acidic groups, in order to achieve elution, enantioseparation and a satisfying peak shape [9].

In polar organic solvent chromatography (POSC), a mixture of basic and acidic additives (*e.g.* diethylamine and trifluoroacetic acid) is occasionally used in the mobile phase when screening for enantioselectivity. For POSC, this dual addition is claimed to yield better results, in addition to the minimization of their memory effect on the stationary phase [20]. In this paper, the use of mixtures of isopropylamine (IPA) and trifluoroacetic acid (TFA) as mobile phase additives with MeOH as modifier in CO_2-based supercritical fluid chromatography is discussed. A test set of 59 pharmaceutical racemates was selected for this purpose. The chiral test set was composed with the intention to cover a broad range of chemically and pharmacologically different pharmaceutical compounds.

2. Materials and methods

2.1. Chemicals

Carbon dioxide 2.7 (purity ≥99.7%) was obtained from Linde Gas® (Grimbergen, Belgium), methanol, HPLC grade, was from Fisher Chemical® (Loughborough, Leicestershire, UK), isopropylamine and trifluoroacetic acid were from Aldrich® (Steinheim, Germany). For the screening of enantioselectivity at given conditions, a test set of 59 racemates was used, obtained from different sources (Table 1).

2.2. Chiral columns

Lux® Cellulose-1 (LC-1) with chiral selector cellulose tris(3,5-dimethylphenylcarbamate), Lux® Cellulose-2 (LC-2) with cellulose tris(3-chloro-4-methylphenylcarbamate), Lux® Cellulose-4 (LC-4) with cellulose tris(4-chloro-3-methylphenylcarbamate), and Lux® Amylose-2 (LA-2) with amylose tris(5-chloro-2-methylphenylcarbamate) were purchased from Phenomenex® (Utrecht, The Netherlands). Dimensions for all columns were 250 mm × 4.6 mm i.d. with 5 μm particle size.

Table 1
Compounds in the screening test set.

Compound	Manufacturer
Acebutolol	Sigma Aldrich, Steinheim, Germany
Acenocoumarol	Novartis, Basel, Switzerland
Alprenolol	Sigma Aldrich, Steinheim, Germany
Ambucetamide	Janssen Pharmaceutica, Beerse, Belgium
Atenolol	Sigma Aldrich, Steinheim, Germany
Atropine	Sigma Aldrich, Steinheim, Germany
Betaxolol	Sigma Aldrich, Steinheim, Germany
Bisoprolol	Origin unknown
Bopindolol	Sandoz, Holskirchen, Germany
Bupranolol	Schwarz Pharma, Monheim, Germany
Carazolol	Astellas Pharma, Munchen, Germany
Carbinoxamine	Origin unknown
Carvedilol	Boehringer, Mannheim, Germany
Chlorphenamine	Sigma Aldrich, Steinheim, Germany
Chlorothalidone	Sigma Aldrich, Steinheim, Germany
Dimethindene	Novartis, Basel, Switzerland
Ephedrine	Sigma Aldrich, Steinheim, Germany
Esmolol	Du Pont de Nemours, Saconnex, Switzerland
Fenoprofen	Sigma Aldrich, Steinheim, Germany
Flurbiprofen	ICN Biomedicals, OH, USA
Hexobarbital	Origin unknown
Ibuprofen	Sigma Aldrich, Steinheim, Germany
Isothipendyl	Origin unknown
Ketoprofen	Sigma Aldrich, Steinheim, Germany
Labetalol	Sigma Aldrich, Steinheim, Germany
Leucovorin	Cynamid Benelux, Brussels, Belgium
Mandelic acid	Sigma Aldrich, Steinheim, Germany
Mebeverine	Duphar, Amsterdam, The Netherlands
Mepindolol	Origin unknown
Meptazinol	Origin unknown
Methadone	Federa, Brussels, Belgium
Methotrexate	Cyanamid Benelux, Brussels, Belgium
Metoprolol	Astra Hassle AB, Lund, Sweden
Mianserine	Diosynth & Organon, Brussels, Belgium
Nadolol	Sigma Aldrich, Steinheim, Germany
Naproxen	Sigma Aldrich, Steinheim, Germany
Naringenin	Sigma Aldrich, Steinheim, Germany
Nicardipine	UCB, Brussels, Belgium
Nimodipine	Bayer, Leverkusen, Germany
Nisoldipine	Bayer, Leverkusen, Germany
Nitrendipine	Bayer, Leverkusen, Germany
Oxazepam	Sigma Aldrich, Steinheim, Germany
Oxprenolol	Cynamid Benelux, Brussels, Belgium
Pindolol	Sigma Aldrich, Steinheim, Germany
Praziquantel	Sigma Aldrich, Steinheim, Germany
Procyclidine	Sigma Aldrich, Steinheim, Germany
Promethazine	Sigma Aldrich, Steinheim, Germany
Propiomazine	Origin unknown
Propranolol	Fluka, Neu-Ulm, Switzerland
Salbutamol	Glaxo Wellcome, Genval, Belgium
Salmeterol	Glaxo Wellcome, Genval, Belgium
Sotalol	Merck, Darmstadt, Germany
Sulpiride	Sigma Aldrich, Steinheim, Germany
Suprofen	Sigma Aldrich, Steinheim, Germany
Terbutaline	Astra-Draco, Lund, Sweden
Tertatolol	Servier Technology, Suresnes, France
Tetramisole	Sigma Aldrich, Steinheim, Germany
Verapamil	Fluka, Neu-Ulm, Switzerland
Warfarine	Sigma Aldrich, Steinheim, Germany

2.3. SFC instrumentation

An analytical system from Waters® (Milford, MA, USA) was used, consisting of a Thar® SFC fluid delivery module (a liquid CO_2 pump and a modifier pump with a 6 solvent switching valve), a Thar® autosampler with a 48-vial plate, a Thar® SFC analytical-2-prep oven with a 10-column selection valve, a Thar® SFC automated backpressure regulator SuperPure Discovery Series, a Waters® 2998 photodiode array detector and a Thermo Scientific® cooling bath type Neslab RTE7 controlled by a Digital One thermoregulator. The instrument is controlled by the software Superchrom® and data

are processed using the Chromscope® software, both from Thar Technologies® (Pittsburgh, PA, USA).

2.4. Chromatographic screening conditions

The test set consisted of 59 racemates prepared in methanol at a concentration of 0.5 mg/ml. Exceptions were leucovorin which was dissolved in water and methotrexate for which MeOH + 0.5% trifluoroacetic acid was used as a solvent due to solubility issues. The compounds were screened on the four chiral stationary phases using four different mobile phase compositions, described in Table 2. Further on, all mobile phase compositions will be expressed in volume-percentages (%).

All experiments were performed using the following conditions: a flow rate of 3.0 ml/min, a temperature of 30 °C, backpressure of 150 bar and analysis time of 30 min. For each enantioseparation, the resolution was calculated using the European Pharmacopoeia method [21]. All compounds that are separated with a resolution (Rs) above 0 are considered (partially) separated, if the resolution is above 1.5 they are considered baseline separated. Compounds that do not elute within the predefined analysis time of 30 min are marked as non-eluting (NE). Compounds with multiple chiral centers, consisting of more than one enantiomeric pair, are considered as separated when at least two enantiomers are (partially or baseline) separated from each other and from the other diastereomers. This applies for labetalol and nadolol, both consisting of two enantiomeric pairs.

2.5. MS operating conditions

The mass spectrometry (MS) analysis was carried out on a Q-Tof micro Mass spectrometer from Waters® (Milford, MA, USA). Direct infusion of the diluted sample was done with a Hamilton syringe pump (Bonaduz, Switzerland). MS conditions were as followed: capillary voltage: 2000 V, sample cone voltage: 25 eV, collision energy: 3 eV, source temperature: 80 °C, desolvation temperature: 120 °C and desolvation gas (nitrogen): 300 l/h. For analysis, an aliquot of the gel-like residue (see Section 3) was diluted with a mixture of 50/50/0.1 (v/v/v) milliQ water/methanol/formic acid.

3. Results and discussion

This study evaluates four polysaccharide-based CSPs (Lux® Cellulose-1, Lux® Cellulose-2, Lux® Cellulose-4 and Lux® Amylose-2) and methanol-containing mobile phases in an attempt to improve efficiency and/or throughput capacity of an existing chiral SFC-screening step. This existing screening, defined by Maftouh et al. [13], uses four coated polysaccharide-based CSPs and one methanol-containing mobile phase, 90/10 (v/v) CO_2/(MeOH + 0.5% IPA or TFA). This implies that compounds are divided into two groups for this screening: one composed of basic, neutral and amphoteric, and one of acidic compounds. Each group is screened with a different additive in the mobile phase, isopropylamine for the first group of compounds, and trifluoroacetic acid for the second.

A mobile phase containing a higher percentage of methanol was investigated secondly (mobile phase B, Table 2). Higher methanol percentages increase the mobile phase strength and will result in faster elution and thus shorter analysis times. If the methanol content can be increased from 10 to 20% without compromising the number of separated compounds (within 30 min), a faster screening might be possible. When switching from mobile phase A to B, an overall increase of five successful separations on Lux® Cellulose-1, of twenty on Lux® Cellulose-2, of eleven on Lux® Cellulose-4 and of one on Lux® Amylose-2 were seen (Fig. 1). Thus, the mobile phase

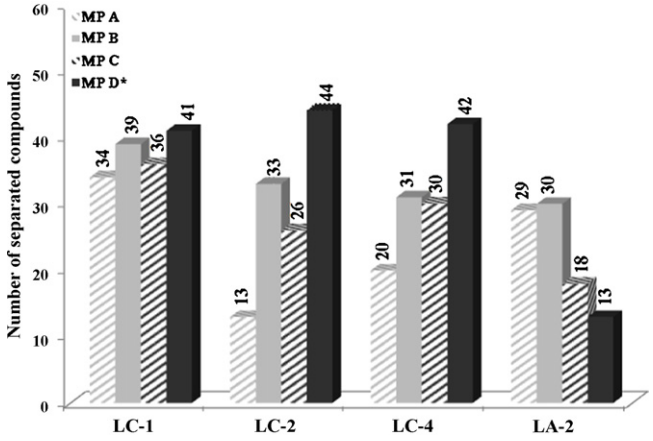


Fig. 1. Screening results expressed as number of separated compounds (out of the test set of 59 compounds) for mobile phases A, B, C and D* on Lux® Cellulose-1 (=LC-1), Lux® Cellulose-2 (=LC-2), Lux® Cellulose-4 (=LC-4), Lux® Amylose-2 (LA-2).

with 20% methanol achieves a higher success rate on all CSPs. This result might sound a bit contradictory, since increasing the modifier concentration will enhance the competition between the modifier and the analyzed compounds for interaction with the stationary phase and thus reduce enantioseparation. However, higher modifier contents lead to a higher elution strength of the mobile phase, implying that more compounds will elute within 30 min when using 20% methanol in the mobile phase. Consequently, it is possible to obtain overall more successful separations within a given analysis time when using higher modifier concentrations, which was here the case. However, using mobile phase B also resulted in the loss of three partial separations on LC-1, of four partial separations on LC-4 and of two partial separations on LA-2. Nevertheless, a net increase in number of successful separations was seen on all CSPs with mobile phase B, compared to mobile phase A.

To explore the possibility of simplifying the screening step and using the same mobile phase for all compounds independent of their chemical properties, methanol-containing mobile phases with both IPA and TFA were tested (MP C and D). However, instrumental problems occurred when screening with mobile phase D, which contains 80% CO_2 and 20% MeOH/IPA/TFA (100/0.25/0.25, v/v/v). The pressure on the system significantly increased and, related to this, an increased pressure drop over the chiral stationary phase was observed. After visual examination, the cause of this problem seemed related to the occurrence of salt precipitation within the system. The only possible origin of this salt is from the mobile phase components. We hypothesized that a salt complex is formed between IPA and TFA. To investigate this hypothesis, three samples of mobile phase D were collected during 1 h after passage through the SFC system and evaporated under a nitrogen flow. The experiments were performed with a co-solvent flow of 0.6 ml/min and a total flow of 3.0 ml/min. The mobile phase residuals were then quantitatively transferred into tarred vials, using a few acetone drops. The residual solvent in the vials was again evaporated under a nitrogen flow and subsequently the vials were dried to constant weight in an oven at 60 °C. After drying, a gel-like residue was found in all vials with an average weight of 94 mg.

To identify the nature of this gel-like substance, an MS analysis was carried out (Fig. 2). This analysis confirmed our hypothesis of salt formation between isopropylamine and trifluoroacetic acid. Several protonated complexes were detected during the MS-analysis, *i.e.* at *m*/*z* 233, 406, 579, 753, 926 and 1099. The signal at *m*/*z* 233 is caused by two isopropylamine moieties forming a protonated complex with one trifluoroacetic acid molecule. The most abundant signal, at *m*/*z* 406, is arising from a protonated

Table 2
Chromatographic screening conditions.

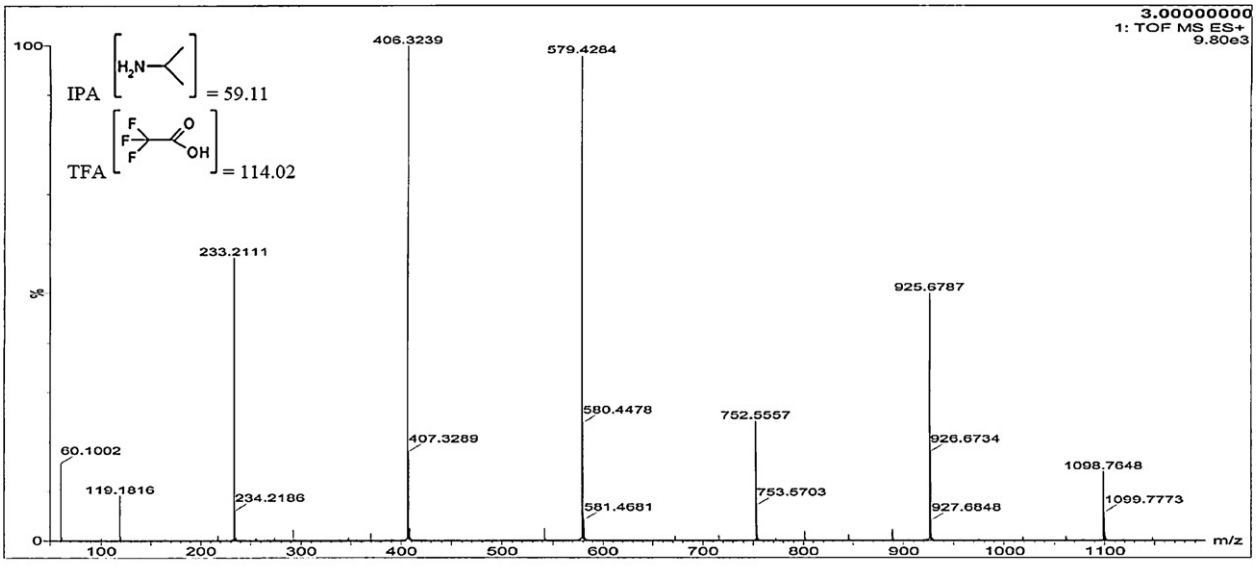
Columns	Lux® Cellulose-1, Lux® Cellulose-2, Lux® Cellulose-4 and Lux® Amylose-2
CO_2-based mobile phases	A: 10% MeOH with 0.5% IPA for basic, neutral and amphoteric compounds or 0.5% TFA for acidic compounds B: 20% MeOH with 0.5% IPA for basic, neutral and amphoteric compounds or 0.5% TFA for acidic compounds C: 10% MeOH with 0.25% IPA and 0.25% TFA for all compounds D: 20% MeOH with 0.25% IPA and 0.25% TFA for all compounds D*: 20% MeOH with 0.10% IPA and 0.10% TFA for all compounds
Temperature	30 °C
Back pressure	150 bar
Analysis time	30 min
Flow rate	3.0 ml/min
Detection	UV detection at 220 nm
Injection volume	5 μl

complex consisting of three isopropylamine and two trifluoroacetic acid moieties, while by adding one extra IPA and TFA unit a complex is formed with *m*/*z* of 579, which also raises an abundant signal in the spectrum. Protonated complexes formed between five IPA and four TFA moieties, between six IPA and five TFA moieties, and between seven IPA and six TFA moieties also appear, though less abundantly.

No precipitation is observed when adding the additives IPA and TFA to the modifier, methanol. However, when mixing 20% of methanol containing 0.25% IPA and 0.25% TFA, with the supercritical and non-polar carbon dioxide, precipitation occurred within the system. This problem did not occur when using only 10% of modifier, and also containing 0.25% IPA and 0.25% TFA, indicating that the complexes are not formed or that the concentration of the formed salt complexes remains below the solubility limit in that mobile phase. For the above reasons we decreased the additive concentrations in 20% MeOH to 0.1% IPA and 0.1% TFA (mobile phase D*, Table 2). Using this adjusted mobile phase, precipitation problems of the IPA–TFA complexes were no longer observed and more importantly, peak tailing was still supressed (Fig. 3).

The resolutions of the screening experiments with mobile phases D and D* are shown in Table 3.

The impact of reducing the additives concentration in the mobile phase on the enantioselectivity of the CSPs was relatively limited. The changes in resolution were reasonably limited. Only for six separations a considerable change in resolution ($\Delta > 1$) was observed: pindolol, acenocoumarol and warfarine on Lux® Cellulose-1, acenocoumarol on Lux® Cellulose-2, and ambucetamide on Lux® Cellulose-2 and Lux® Cellulose-4. However, as the resolution of these separations remained significantly higher than 1.5, no baseline separations were lost when changing from mobile phase D to D*. These results indicate that the overall



Identification : $\left[\text{TFA}\right]_n\left[\text{IPA}\right]_m + H^+$

m \ n	1	2	3	4	5	6
1	173.13	287.15	401.17	515.19	629.21	743.23
2	**232.24**	346.26	460.28	574.30	688.32	802.34
3	291.35	**405.37**	519.39	633.41	747.43	861.45
4	350.46	464.48	**578.50**	692.52	806.54	920.56
5	409.57	523.59	637.61	**751.63**	865.65	979.67
6	468.68	582.70	696.72	810.74	**924.76**	1038.78
7	527.79	641.81	755.83	869.85	983.87	**1097.89**

Fig. 2. MS spectrum of the gel-like residue, the masses indicated in bold and underlined are present as protonated species.

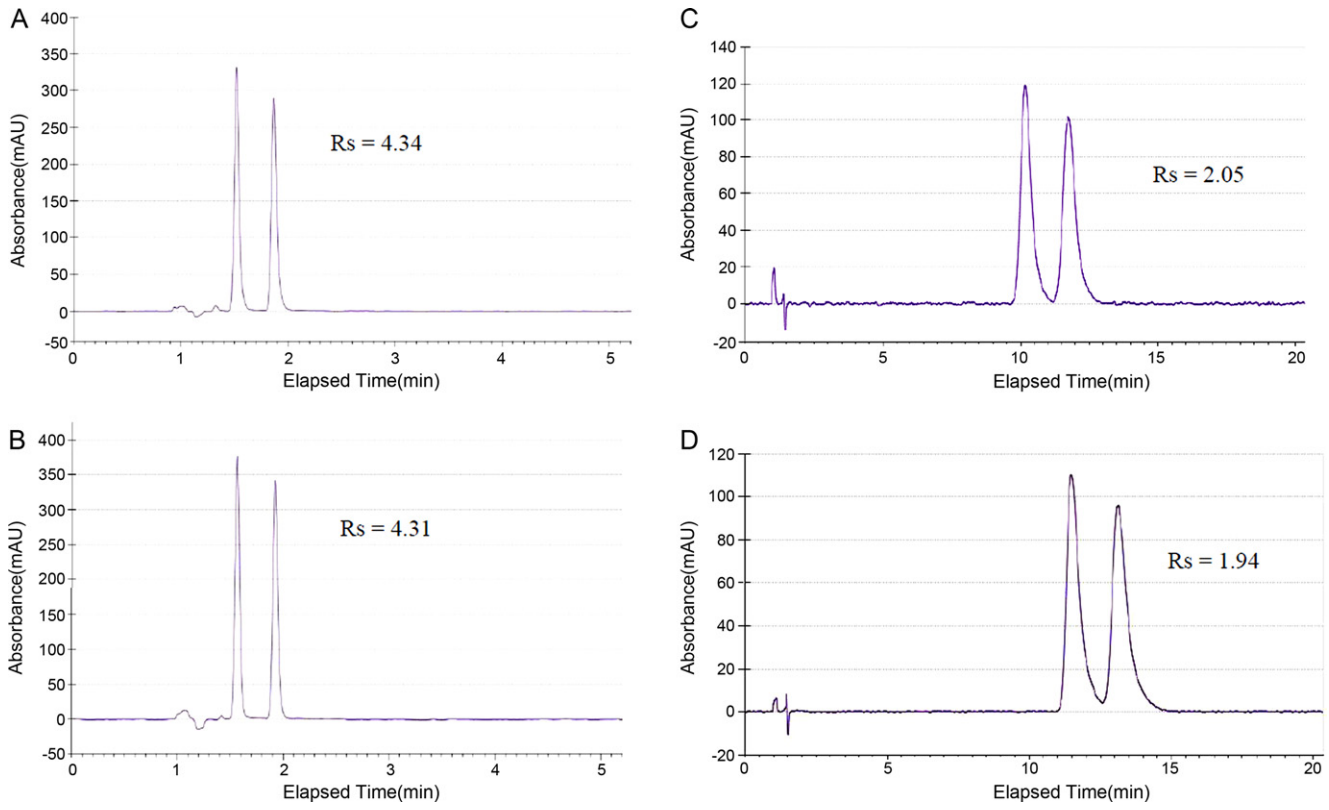


Fig. 3. Chromatograms of alprenolol on Lux® Cellulose-1 with (A) 20% MeOH + 0.25% IPA + 0.25% TFA and (B) 20% MeOH + 0.1% IPA + 0.1% TFA mobile phases and of carazolol on Lux® Cellulose-2 with (C) 20% MeOH + 0.25% IPA + 0.25% TFA and (D) 20% MeOH + 0.1% IPA + 0.1% TFA mobile phases.

enantioselectivity was similar for mobile phases D and D*, but mobile phase D* offered the advantage of avoiding instrumental problems. Thus D* was used as screening mobile phase for further experiments.

Mobile phases C and D* contain both isopropylamine and trifluoroacetic acid as additives, but with 10 and 20% methanol respectively. When comparing the successful separations changing from MP C to D* (Fig. 1) an increase of five separations on LC-1, of eighteen separations on LC-2 and of twelve separations on LC-4 was seen. On LA-2 mobile phase D* generated five separations less than mobile phase C. This implies that a mobile phase with higher solvent strength, and thus a higher methanol-content, is more appropriate for the cellulose-based stationary phases when two additives are added since MP D* generated more separations than MP C within the predefined analysis time of 30 min. As mentioned earlier, the increased rate of successful separations is due to the fact that more compounds will elute within the fixed analysis time of 30 min. For Lux® Amylose-2, MP C was more successful than MP D*. However, both are much less successful than MP A and B, containing separate additives with respectively 10 and 20% methanol. The combined use of the additives here caused a large decrease in retention and therefore in enantioselectivity.

From Fig. 1 it can also be observed that mobile phase D* on the cellulose columns gives an increased success rate compared to the equivalent mobile phase with the same percentage of methanol but with only one additive (MP B). An increase of two separations on LC-1, and of eleven on LC-2 and LC-4 were seen. The supplementary separations obtained using mobile phase D* instead of B, were exclusively basic, neutral or amphoteric compounds. The separation of nimodipine and terbutaline on LC-1, of acebutolol, meptazinol and propranolol on LC-2, and of propranolol and promethazine on LC-4 was lost when switching from MP B to D*. For the acidic compounds, no change in enantioselectivity was seen on the cellulose columns, when switching from MP B to D*.

On LA-2 a global decrease of seventeen separations was seen when changing from MP B to D*, again almost entirely due to the non-acidic compounds. This implies that the combination of isopropylamine and trifluoroacetic acid has a deleterious effect on the retention and separation on LA-2.

Average analysis times were shorter with mobile phase D* compared to mobile phase B on all CSPs. For LC-1 the average decrease of 0.2 min was rather limited, but for the other CSPs the average decreases of 4.6, 4.6 and 4.9 min for LC-2, LC-4 and LA-2 respectively were more pronounced. The combined use of IPA and TFA in 20% methanol thus led to shorter analysis times on all stationary phases. This mobile phase displayed a broader enantioselectivity on the cellulose-based phases because more compounds eluted within the acceptable run time of 30 min. It decreased largely the enantioselectivity on the amylose-based stationary phase, because for many compounds the retention has disappeared or was too limited.

These results illustrate that the effect of additives is also dependent on the selectors on the stationary phase. They consist of helical derivatised polysaccharide chains with internal carbamate and external aromatic groups. For LC-1, LC-2 and LC-4, cellulose forms the backbone of the stationary phase, while derivatised amylose is used in LA-2. Structural differences in the helical structures of cellulose and amylose might result in different interactions with the additives in the mobile phase, resulting in different effects on the enantioselectivity exhibited by the CSP. However, the influence of additives on the polysaccharide-based stationary phases remains complex and is to date not fully elucidated yet [22]. We also observed that the optimal mobile phase solvent strength is different for different selectors. Combined with the addition of additives it might lead to a worsened situation rather than to an improved enantioselectivity, as was seen for LA-2.

Table 3
Resolutions from the screening on Lux® Cellulose-1, Lux® Cellulose-2, Lux® Cellulose-4 and Lux® Amylose-2 with mobile phases D: 80/20 (v/v) CO_2/(MeOH + 0.25% IPA + 0.25% TFA) and D*: 80/20 (v/v) CO_2/(MeOH + 0.10% IPA + 0.10% TFA). Conditions: 3 ml/min, 30 °C, 150 bar backpressure, 5 μl injection volume, detection at 220 nm.

	Column	Lux® Cellulose-1		Lux® Cellulose-2		Lux® Cellulose-4		Lux® Amylose-2	
	Mobile phase	D	D*	D	D*	D	D*	D	D*
Basic, neutral and amphoteric compounds									
1	Acebutolol	1.28	1.39	0.00	0.00	0.00	0.00	0.00	0.00
2	Alprenolol	4.34	4.31	1.35	1.35	1.32	1.29	0.00	0.00
3	Ambucetamide	0.67	1.20	9.18	15.47	24.59	17.20	0.00	0.00
4	Atenolol	13.27	12.47	0.00	0.00	0.00	0.00	0.00	0.00
5	Atropine	2.73	2.88	1.43	1.38	0.00	0.00	0.00	0.00
6	Betaxolol	16.08	15.78	1.33	1.29	1.61	1.81	0.00	0.00
7	Bisoprolol	10.63	10.97	1.30	0.67	1.37	1.33	0.00	0.00
8	Bopindolol	27.64	28.71	10.57	9.92	9.22	9.47	0.00	0.00
9	Bupranolol	1.25	1.37	1.54	1.42	1.93	2.04	0.00	0.00
10	Carazolol	4.59	4.01	2.05	1.94	2.52	2.85	0.00	0.00
11	Carbinoxamine	0.00	0.00	2.48	2.24	3.36	3.08	0.00	0.00
12	Carvedilol	N.E.	N.E.	N.E.	N.E.	1.01	1.61	0.21	0.23
13	Chlorphenamine	0.37	0.52	0.64	0.64	1.46	1.26	0.00	0.00
14	Chlorthalidon	4.85	4.72	5.13	6.13	0.00	0.00	1.57	1.53
15	Dimethindene	7.27	6.93	1.47	1.29	1.73	1.61	0.00	0.00
16	Ephedrine	0.67	1.38	2.89	2.97	4.08	3.91	0.00	0.00
17	Esmolol	13.37	12.82	1.35	1.29	1.30	1.35	0.00	0.00
18	Isothipendyl	1.37	1.26	0.60	0.54	0.52	0.31	0.00	0.00
19	Labetalol – pair 1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	Labetalol – pair 2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
20	Mebeverine	1.85	1.80	1.63	1.63	0.66	0.63	0.00	0.00
21	Mepindolol	0.00	0.00	7.45	7.12	6.67	6.98	0.00	0.00
22	Meptazinol	5.44	5.26	0.00	0.00	0.48	0.24	0.00	0.00
23	Methadone	0.59	0.57	1.64	1.56	1.30	1.28	0.00	0.00
24	Metoprolol	13.97	14.28	1.33	1.35	1.55	1.78	0.00	0.00
25	Mianserine	2.58	2.73	2.28	2.02	4.79	5.50	0.00	0.00
26	Nadolol – pair 1	3.55	3.36	1.38	1.52	0.12	0.43	0.00	0.00
	Nadolol – pair 2	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
27	Nicardipine	0.00	0.00	1.37	1.33	3.63	4.23	0.00	0.00
28	Nimodipine	0.00	0.00	0.67	0.67	0.00	0.00	0.00	0.00
29	Nisoldipine	0.00	0.00	1.78	1.84	1.65	1.68	0.00	0.00
30	Nitrendipine	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
31	Oxazepam	12.02	11.31	2.44	2.50	3.95	3.90	1.10	0.96
32	Oxprenolol	9.61	9.24	0.50	0.53	0.19	0.26	0.00	0.00
33	Pindolol	25.13	23.64	6.74	6.36	6.51	6.64	0.00	0.00
34	Praziquantel	2.80	2.94	7.02	7.06	7.66	7.74	3.51	3.89
35	Procyclidine	2.20	2.14	1.30	1.33	3.21	2.30	0.00	0.00
36	Promethazine	0.51	0.47	1.35	1.36	0.00	0.00	0.00	0.00
37	Propiomazine	0.00	0.00	0.70	0.66	0.00	0.00	0.32	0.60
38	Propranolol	11.78	10.75	0.54	0.00	0.00	0.00	0.00	0.00
39	Salbutamol	0.00	0.00	3.75	3.41	4.04	3.87	0.00	0.00
40	Salmeterol	0.63	1.25	4.95	4.47	5.14	5.31	0.00	0.00
41	Sotalol	0.62	0.63	1.65	1.51	1.99	2.64	0.00	0.00
42	Sulpiride	2.83	2.51	N.E.	N.E.	0.00	0.39	0.00	0.00
43	Terbutaline	0.00	0.00	6.36	6.48	9.10	9.32	0.00	0.00
44	Tertatolol	9.60	9.10	5.26	4.71	2.92	3.06	0.00	0.00
45	Tetramisol	2.93	2.26	3.59	3.18	4.26	3.24	0.00	0.00
46	Verapamil	3.34	3.12	1.46	1.52	3.33	3.40	0.00	0.00
47	Methotrexate	0.00	0.00	0.89	1.04	1.12	1.09	0.00	0.00
Acidic compounds									
1	Acenocoumarol	7.45	5.45	3.57	4.66	0.00	0.00	1.15	0.95
2	Fenoprofen	0.00	0.00	0.00	0.00	0.00	0.32	1.27	1.40
3	Flurbiprofen	0.00	0.00	0.00	0.00	0.00	0.00	3.64	3.57
4	Hexobarbital	1.62	1.68	7.34	7.22	6.82	7.06	2.12	2.24
5	Ibuprofen	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
6	Ketoprofen	0.00	0.00	0.00	0.00	0.00	0.00	1.18	1.58
7	Leucovorin	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
8	Mandelic acid	0.00	0.00	0.00	0.00	0.00	0.00	2.22	2.22
9	Naproxen	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
10	Naringenin	1.93	1.97	3.52	3.48	2.75	2.74	1.00	1.31
11	Suprofen	1.32	0.66	1.35	1.36	0.40	0.44	0.00	0.00
12	Warfarine	12.06	14.64	3.53	3.65	3.40	3.59	5.00	5.15

Additives exert their effect on enantioselectivity through different mechanisms. Isopropyl- and other amines decrease non-stereospecific interactions by shielding residual silanol groups and other non-specific binding sites, and by suppressing the ionization of compounds. Hereby, enantioselective interactions as retention mechanism are favored [17,20,23]. Additionally, separation efficiency improves when adding small amounts of isopropylamine to the mobile phase, by facilitating adsorption and desorption of basic compounds from the polysaccharide-based stationary phase [17].

As acidic additive, trifluoroacetic acid improves enantioselectivity by enhancing hydrogen bonding interactions between the amino groups of the compounds with carbonyl groups of the carbamate functions of the polysaccharide-based selectors. This is

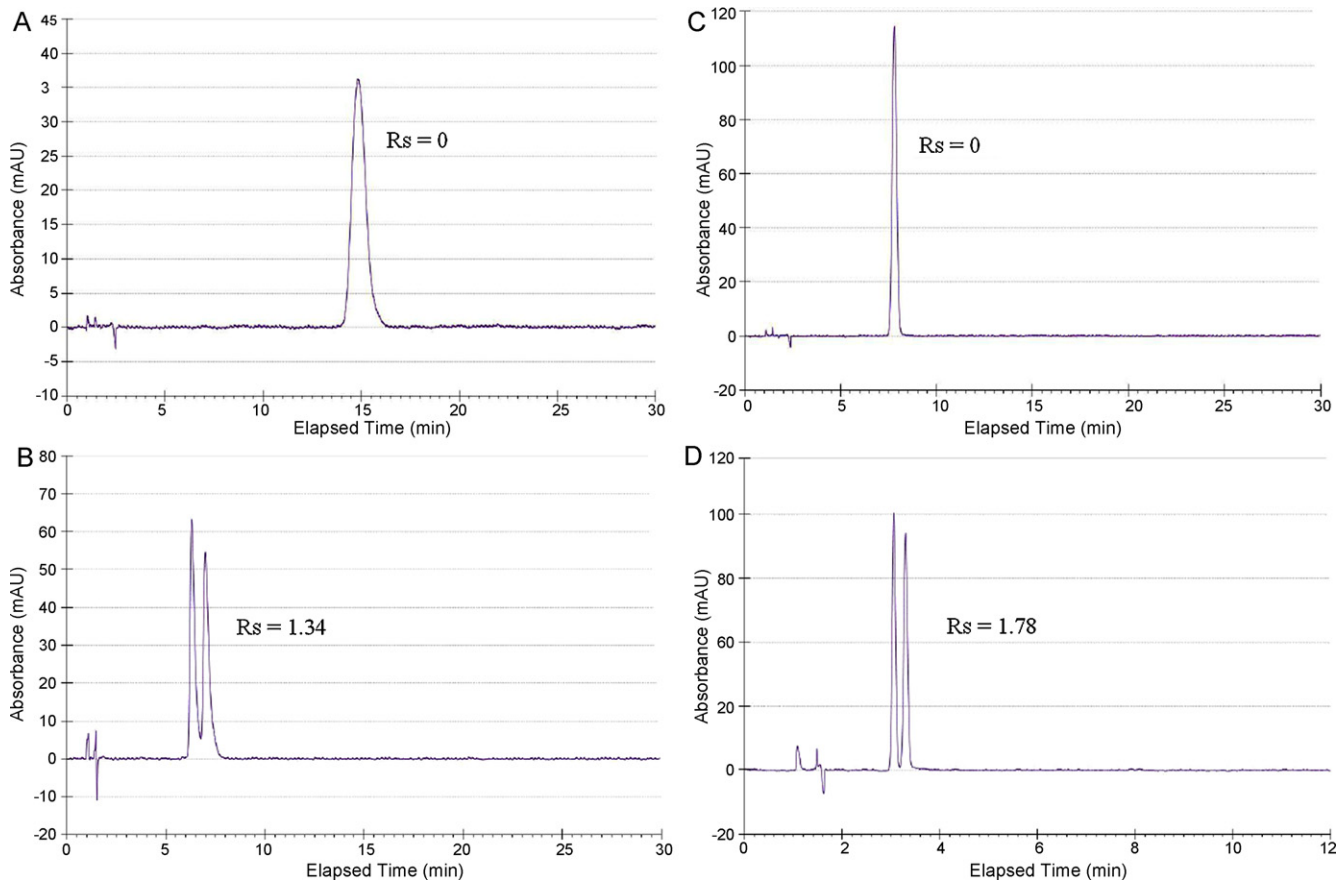


Fig. 4. Chromatograms of methadone on Lux® Cellulose-1 with (A) 20% MeOH:IPA and (B) 20% MeOH:IPA:TFA mobile phase, and of metoprolol on Lux® Cellulose-4 with (C) 20% MeOH:IPA and (D) 20% MeOH:IPA:TFA mobile phases.

achieved by a protonation of functional amino groups of the analyzed compounds [24]. Addition of TFA to the mobile phase reduces the pH and the ionization of acidic compounds, resulting in an enhanced enantioselectivity. Trifluoroacetic acid may also form salts with charged basic compounds. These salts can interact better with the stationary phase and be separated intact in supercritical fluid chromatography [23]. However, the salt with the basic additive can also cause problems as was observed in this study.

The effects that additives provoke, are even more complex due to the presence of carbon dioxide in the mobile phase. Transient complexes are formed between carbon dioxide and amino groups of compounds. These complexes interact as such with the stationary phase. Basic additives, such as isopropylamine, cause a deprotonation of functional amino groups, which results in a reduction of this complex formation [23]. Thus, isopropylamine and trifluoroacetic acid can both influence enantioselectivity, as individual additives, through the above mentioned mechanisms. As isopropylamine enhances enantioselectivity of basic compounds and trifluoroacetic acid of both acidic and basic compounds, a combination of these additives can lead to higher separation rates for a diverse chiral test set, as was seen in our study.

By combining isopropylamine and trifluoroacetic acid, interactions become even more complicated. Our experiments showed the formation of salt complexes between isopropylamine and trifluoroacetic acid. However, most probably an equilibrium exist between uncomplexed IPA and TFA and the IPA–TFA complexes. On the other hand, pH also plays an important role in compound ionization and enantioselective interactions. Because the complexed and uncomplexed fractions of the additives is unknown, their residual effect on the pH is therefore unpredictable. In addition, carbon dioxide itself has an acidic nature in the presence of protic modifiers, such as methanol [19].

It is clear that many potential interactions are influenced when additives are used in the mobile phase. Different mechanisms, which to date are not all fully understood, can influence the enantioselectivity. Our experiments pointed out that a combination of isopropylamine and trifluoroacetic acid enhanced the overall enantioselectivity of the evaluated cellulose-based phases, while it decreased the overall number of successful separations on the amylose-based phase. These results indicate that in SFC it can be useful to screen with mobile phases containing both isopropylamine and trifluoroacetic acid additives since a significantly different and broader enantioselectivity can occur compared to using only one additive. This is illustrated in Fig. 4 for methadone and metoprolol. However, in SFC salt formation between IPA and TFA, occurring when the additives and the MeOH concentrations are too high, should be avoided.

4. Conclusions

In chiral supercritical fluid chromatography we observed a different and, in most cases, broader enantioselectivity on polysaccharide-based CSPs when using simultaneously IPA and TFA as additives compared to the separate use of these additives in the mobile phase. However, when working with a combination of these two additives, one should avoid the possible formation and precipitation of salt complexes when mixing the modifier with the supercritical carbon dioxide. When using 20% of methanol, containing 0.25% isopropylamine and 0.25% trifluoroacetic acid in

supercritical carbon dioxide, instrumental problems occurred, due to precipitation of salt-complexes, formed by the mobile phase additives. Reducing the additive concentrations to 0.1% for both IPA and TFA solved this problem, while the overall success rate and peak shapes were practically uninfluenced.

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