Journal of Chromatography A, 1218 (2011) 74–82

Contents lists available at ScienceDirect

Journal of Chromatography A

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

Orthogonal analytical screening for liquid chromatography–mass spectrometry method development and preparative scale-up

Luis M. Font [a,*], A. Fontana [a], M.T. Galceran [b], L. Iturrino [a], V. Perez [a]

[a] *Enabling Analytical Technologies, Janssen Research & Development a Division of Janssen-Cilag S.A., c/Jarama 75, 45007 Toledo, Spain*
[b] *Departament de Química Analítica, Facultat de Química, Universitat de Barcelona, Diagonal, 647, 3ª planta, 08028 Barcelona, Spain*

ARTICLE INFO

Article history:
Received 22 February 2010
Received in revised form 1 October 2010
Accepted 26 October 2010
Available online 2 November 2010

Keywords:
HPLC–MS
HPLC method development
Preparative HPLC–MS
Orthogonal screening

ABSTRACT

An analytical HPLC–MS screening methodology has been developed to improve preparative RP-HPLC–MS purifications in medicinal chemistry laboratories. Although several approaches have been previously described to optimize analytical separations, none of them met our needs for the optimization of preparative conditions. Our screening protocol is based on searching among several orthogonal conditions to find the optimum preparative separation. Five different buffer conditions, from low to high pH, two organic solvents, acetonitrile and methanol, and five stationary phases of different polarities and characteristics were used. The orthogonality of the system was demonstrated using both, a standard mixture and mixtures from synthesis. To carry out the screening one of the analytical "open access" HPLC–MS systems was modified to perform the analytical screening while maintaining the open-access functionality for synthesis reaction monitoring. A software tool for automated sample programming and data reporting was also developed.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

Preparative RP-HPLC–MS is one of the most powerful techniques currently used for the purification of synthesized compounds in medicinal chemistry laboratories. Today, the requirements for this technique are quite different from the initial demands of the combinatorial chemistry libraries. Instruments and methodologies were first developed to satisfy the requirements of the purification of large series of compounds [1,2] and in this context, RP-HPLC–MS was able to remove the unwanted reagents or side products that other techniques such as scavenger resins [3], liquid–liquid [4] or solid phase extractions [5] were not able to eliminate. On the other hand, the compound requirements for pharmacological tests changed as we moved from the hit to lead (HTL) phase to the lead optimization (LO) phase in the drug discovery process. The number of compounds in the lead optimization stages of the discovery process is lower compared to the HTL phase, but the information required for each compound is higher. For this reason, not only the purity but also the amount of purified compound is important since a larger quantity is required for the studies being performed at this stage [6,7]. This is reflected in the increasing importance of having an efficient chromatographic procedure to purify compounds of pharmacological interest [8,9].

To separate mixtures of a wide range of polarities it is quite common to use standard generic gradients which can be applied efficiently and allow for increased laboratory throughput [1]. In general, the results are satisfactory and compounds are recovered with a high purity. However, frequently standard methods do not deliver sufficient separation for some crude mixtures. For instance, the presence of diastereoisomers, regioisomers and/or impurities which have small chemical differences compared to the main compound often results in closely eluting peaks in the chromatogram which are not well resolved with standard methods. Moreover, in recent years, the increased degree of automation and performance in normal phase low pressure purification systems has allowed the purification of many compounds by this technique in our laboratory. As a result, most compounds purified by our preparative RP-HPLC–MS instruments are the most challenging samples not resolved by standard methods.

The strategy of HPLC method development used in medicinal chemistry laboratories must fulfill several conditions. The first is that the methodology should lead to orthogonal results. This means that the use of different experimental conditions such as different columns or mobile phases, should give different retention times and elution orders in order to provide the desired separation [10]. With reversed phase HPLC different orthogonal experimental conditions can be achieved, as well as conditions for method development, such as changing the organic phase, the pH, the stationary phase and the temperature [11,12].

In order to find the optimal separation method for a given sample the screening of a wide range of conditions must be performed.

* Corresponding author. Tel.: +34 925 24 57 85; fax: +34 925 24 57 71.
E-mail address: lfont@its.jnj.com (L.M. Font).

0021-9673/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2010.10.102

Method screening has been used in the field of early drug discovery, for applications such as purity assessment. For instance, it has been found that the use of orthogonal separation procedures reduces the risk of incorrect purity assessment of drug compounds and ensures the quality and safety of drug products. This can be achieved by changing pH, organic solvents, aqueous buffer and stationary phases [13–16]. Several elegant approaches have been used to automate this method searching. For example, the fine optimization of pH done by Loeser et al. [17] and the automated screening of various column and mobile phase combinations done by Hewitt et al. [18]. These works do not use mass spectrometry to identify the compounds and they are not focused to be scaled-up to preparative separations.

Some other method development approaches have been done with the objective of the purifications of chiral compounds of pharmacological interest, Francotte and Wetli, described an automated chiral separation-screening HPLC platform that performs simultaneous column conditioning during on-going analysis [19]. Zeng et al. designed an automated parallel four column SFC-MS system for high throughput enantioselective method development and optimization with a custom Visual Basic program to control the whole process [20]. However, none of these approaches fixed to our need to purify challenging mixtures from synthesis by RP-HPLC–MS in a reduced timeframe. The aim of this work is to establish orthogonal RP-HPLC–MS conditions capable of providing the best separation for the purification of different compounds. For an efficient screening a careful selection of buffers (from low to high pH), organic solvents (acetonitrile or methanol) and stationary phases as well as instrument characteristics is required. Our primary objective was to develop methods capable of being scaled up to preparative conditions. For this reason, temperature modifications were not taken into consideration since it is difficult to control temperature at the high flow rates used in the preparative scale.

The evolution of column technology allows working at low or high pH with no risk for the stability and lifetime of the column. This is of great importance and offers increased flexibility such as when working with high p*K*a compounds a high pH can be used to avoid interactions with free silanols in the column support which would result in narrower peaks. This permits increased loadability of the columns in the preparative runs [21].

In this work we have established an automated set of 20 experiments to be performed overnight and a faster set of 4 experiments to be done during working hours. One of the difficulties faced and solved in this scenario was the equilibration time needed when changing conditions (solvent, buffer and column). Optimization of the software was also needed in order to program samples in an efficient way and to obtain a short and legible report of the results to allow quick decision-making.

Instrument optimization was performed on one of our “open access” analytical HPLC–MS systems. The goal was to keep the instrument in an optimal configuration to perform both the analytical reaction follow up in an “open access” environment for chemists during daily working hours and the analytical screening overnight.

2. Experimental

2.1. Chemicals and materials

LC–MS Chromasolv® acetonitrile and methanol used in the analytical HPLC were supplied by Fluka (Steinheim, Germany). HPLC gradient grade acetonitrile and methanol used in the preparative HPLC were purchased from J.T. Baker (Deventer, The Netherlands). De-ionized water was produced by a MilliQ gradient A10 system from Millipore (Bedford, MA, USA) and was used without further treatment. Ammonia solution (32%) was supplied by Merck (Darmstadt, Germany) and formic acid (98–100%) by Scharlau Chemie (Barcelona, Spain). Ammonium hydrogen carbonate (99.0%), ammonium acetate (99.0%) and ammonium formate (99.0%) were obtained from Fluka (Steinheim, Germany).

Buffers were prepared by weight. Ammonium formate/formic acid buffer pH: 3.1 (0.6 g/L of the formate salt and 2 mL/L of the acid), ammonium hydrogen carbonate pH 7.9 (1 g/L). Ammonium hydrogen carbonate pH 9.7 (1 g/L of salt and ammonia to the desired pH). Ammonium acetate solution used for routine analysis was prepared by adding 50 mL of acetonitrile to a 950 mL ammonium salt solution in water containing 0.05% of the salt. Ammonium acetate solution used for the analytical screening was prepared by adding 4 g of the salt to 1 L of water. Formic acid solution pH 2.4 was prepared by adding 5 mL of formic acid to water up to 1 L of solution.

The analytical columns XBridge C-18 (100 mm × 4.6 mm, 5 μm), XBridge Phenyl (100 mm × 4.6 mm, 5 μm), Sunfire C-18 (100 mm × 4.6 mm, 5 μm), Atlantis C-18 (100 mm × 4.6 mm, 5 μm), Atlantis Hilic (50 mm × 4.6 mm, 5 μm) were supplied by Waters (Mildford, MA, USA) and Eclipse Plus C-18 (30 mm × 4.6 mm, 3.5 μm) was purchased from Agilent Technologies (Agilent Technologies, Waldbronn, Germany).

The preparative columns XBridge C-18 (100 mm × 19 and 30 mm, 5 μm), XBridge Phenyl (100 mm × 19 mm, 5 μm), Sunfire C-18 (100 mm × 19 and 30 mm, 5 μm), Atlantis C-18 (100 mm × 19 mm, 5 μm), Atlantis Hilic (50 mm × 19 mm, 5 μm), were supplied by Waters (Mildford, MA, USA).

The analytical column used in the UPLC system for quality control of purified compounds was a BEH-C18 column (50 mm × 2.1 mm, 1.7 μm) from Waters.

2.2. Standards and sample preparation

Haloperidol was obtained from Janssen Pharmaceutica. Caffeine, p-nitrophenol, chlorotalidone, 3-(α-acetonylbenzyl)-4-hydroxycoumarin, flavone and nabumetone were all commercially available from Sigma–Aldrich (St. Louis, MO). Some characteristics of the compounds are given in Table 1.

The standard mixture used as test for system suitability was prepared by weight adding 3 mg of each compound to 20 mL of methanol. Stock solutions were stored in an amber 30 mL vial refrigerated at −5 °C, for one month without showing significant degradation.

2.3. Instrumentation

2.3.1. Analytical HPLC–MS for method development

An Agilent 1100 HPLC–MS (Agilent Technologies, Waldbronn, Germany) optimized with the Agilent Rapid Resolution Kit was used. It consisted of a binary pump, a degasser, an injector, a column oven with a 6-port switching valve, a diode array (80 Hz) detector (DAD), an analytical solvent valve for 12 solvents and a 6-positions column selector valve. UV detection with a DAD at 325 nm with a bandwidth of 250 nm was used. An additional signal was registered at 254 nm with a bandwidth of 30 nm, to obtain a better baseline when using the formic and formate buffers. The gradient elution program was: a linear gradient from 10% to 100% of organic solvent in 6 min, kept 0.5 min, plus 2 min of equilibration time at initial conditions. For ammonium formate/formic acid and ammonium acetate conditions with acetonitrile solvent using Sunfire column, we have observed empirically that two extra minutes of equilibration time were needed to achieve reproducibility.

Flow was split to a single quadrupole mass spectrometer detector, Agilent MSD, configured with an atmospheric pressure ionization source API, an ESCI multimode ionization source, so that 200–300 μl was introduced to the detector. Electrospray mass spectrometry measurements were performed, acquiring

Table 1
Structure and properties of the compounds.

Compound number	Compound	Structure	Formula	MW (free base)	pKa/pKb
1	Caffeine		$C_8H_{10}N_4O_2$	194.193	pKb = 0.74
2	p-Nitrophenol		$C_6H_5NO_3$	139.109	pKa = 7.23
3	Chlorthalidone		$C_{14}H_{11}ClN_2O_4S$	338.77	pKa = 9.57
4	Hydroxicumarine		$C_{19}H_{16}O_4$	308.331	pKa = 4.5 (OH vinyl/keto tautomery)
5	Haloperidol		$C_{21}H_{23}ClFNO_2$	375.87	pKa = 13.86
6	Flavone		$C_{15}H_{10}O_2$	222.242	–
7	Nabumetone		$C_{15}H_{16}O_2$	228.289	–

simultaneously in both positive and negative ionization modes (fragmentor 70 V, threshold spectral abundance 100, MS peak width 0.1 min, capillary voltage, 1000 V) over the mass range of 100–1000 Da. Nitrogen was used as the nebulizer gas, nebulizer pressure of 60 psig. The source temperature was maintained at 100 °C, drying gas flow of 5.0 L/min and drying gas temperature of 350 °C. An Agilent Chemstation was used for instrument control and data acquisition.

2.3.2. Preparative HPLC–MS

Two preparative HPLC systems were used. The first one was from Agilent Technologies (Agilent Technologies, Waldbronn, Germany) and consisted of a 1100 binary preparative pump, a 1200 analytical quaternary pump used as make-up pump a 1200 dual loop autosampler, an analytical 1100 DAD detector and a MSD detector with ESI ionization source (both used to trigger fraction collection), three 1200 preparative fraction collectors a solvent valve to switch among the collectors, an MRA active splitter from Rheodyne, a 1200 variable wavelength detector with preparative cell, used as recovery collector and an analytical 1200 fraction collector used as waste collector. Electrospray mass spectrometry measurements were performed, acquiring in positive and/or negative ionization modes (fragmentor 70 V, threshold spectral abundance 150, MS peak width 0.1 min, capillary voltage, 2500 V) over the mass range of 100–900 Da. Nitrogen was used as the nebulizer gas, nebulizer pressure of 45 psig. The drying gas flow was of 10.0 L/min and the drying gas temperature of 300 °C. The whole system was controlled by the Agilent Chemstation.

The other one consisted of a 2525 binary preparative pump, a 2767 injector/collector, a 515 analytical pump used as make-up pump, a FCII waste collector, a 996 PDA and a ZQ detector with ESI ionization source (both used to trigger fraction collection) all from Waters (Waters, Midford, MA), a Knauer 2500 Smartline UV detector (Berlin, Germany) with a preparative cell used as waste detector, and a MRA active splitter from Rheodyne (Rohnert Park, CA, US).

Software control was done by MassLynx and FractionLynx. Electrospray mass spectrometry measurements were performed, acquiring in positive and/or negative ionization modes (cone voltage 30 V, multiplier voltage 350, scan time 0.5 s, interscan time

0.1 s, capillary voltage, 2500 V) over the mass range of 150–1000 Da. Nitrogen was used as the nebulizer gas, the nebulizer gas was flow of 350 L/h and the cone gas flow of 10 L/h. The source temperature was 140 °C and the desolvatation temp. was 250 °C.

Both systems were in-house modified to perform "at column dilution" injection. In this injection technique the autosampler is set up in the flow path of the pump delivering the organic mobile phase and the sample is mixed with the aqueous mobile phase immediately before the column. This approach prevents samples from precipitation in the injector pathways and also minimizes problems as peak fronting when injecting samples in strong organic solvent [22,23].

2.3.3. Analytical instrumentation for purity control

For analytical control of final products after preparative HPLC–MS, an analytical Waters Acquity-SQD UPLC–MS system was used, including a binary solvent manager, a sample organizer, a column manager, a PDA detector and a SQD detector. Software control was done by MassLynx and OpenLynx. Reversed phase UPLC was carried out, with a flow rate of 0.8 mL/min, at 60 °C without split to the MS detector. The gradient conditions used are: 90% A (0.5 g/L ammonium acetate solution), 10% B (mixture of acetonitrile/methanol, 1/1), to 100% B in 6.85 min, kept till 7.50 min and equilibrated to initial conditions at 7.75 min until 9.0 min. Low-resolution mass spectra (single quadrupole, SQD detector) were acquired by scanning from 100 to 1000 Da in 0.1 s using an inter-channel delay of 0.08 s. The capillary needle voltage was 3 kV. The cone voltage was 20 V for positive ionization mode and 30 V for negative ionization mode.

^{1}H NMR experiments were performed with a Bruker DPX-400 MHz spectrometer, Dual ATM ("Automatic Tuning & Matching") probe, Z-gradients, variable Tª and B-ACS autosampler. ICONNMR, Topspin and ACD software were used for instrument control and spectra interpretation.

3. Results and discussion

3.1. Instrumental modifications

Several arrangements in one "open access" analytical HPLC–MS system were performed in order to build a flexible configuration able to perform both the analytical "open access" reaction monitoring during the working day and the analytical screening overnight. For this purpose three valves were used: a 12 position/13 port valve for solvent selection, a 2 position/6 port valve to hold the analytical column used for routine work and a 6 position valve for column selection (see Fig. 1).

The use of the 12 position solvent selection valve in combination with the capability of changing between two solvents/buffers of the binary pump opened the range of combinations. The two ports of one of the pump channels were used for the organic solvents acetonitrile and methanol. The solvent selection valve and the other pump channel with two positions were used to choose among six buffers. The 2 position /6 port valve on the column oven was used for selecting the working column for the reactions follow-up or to connect the columns used for screening. Using this set-up it was possible to place the column used for routine work, with smaller particle size, in the oven and in the most optimum pathway getting benefit of both the optimum temperature and the rapid resolution tubing kit. The extra column selection valve hold five columns with different stationary phases, that were used to perform the method screening based on stationary phase selectivity [24]. With this configuration we obtained good reproducibility for all methods except for Sunfire C-18 with ammonium acetate buffer. For this reason the sixth port of this selection valve was used to connect another Sunfire C-18 column that was used for neutral conditions. This approach provided the needed reproducibility.

3.2. Buffer selection

In preparative liquid chromatography pH is a parameter more critical than in analytical liquid chromatography since when overloading the column interactions between the analyte and free silanols of the column support became very important leading to a dramatic tailing effect. To prevent this effect working at pH conditions where the neutral form of the compound is present at the highest percentage is mandatory. So, for acid groups, a pH two units below p*K*a must be used, since at these conditions 99% of the compound is in the protonated neutral form. For basic compounds, working around two units above p*K*a is recommended.

Since many small molecules of pharmacological interest have basic groups in their structure, basic buffers are ideal to provide narrow peaks and as a consequence a high loadability [21,25]. Among buffers, ammonium bicarbonate was selected because it has been previously described as an excellent buffer for HPLC–MS analysis because of its good chromatographic behavior and reproducible separation. In addition, its volatility makes it an appropriate buffer for HPLC–MS using atmospheric pressure ionization sources [26,27]. Its volatility is also of great help when evaporating the samples resulting from a preparative process as it eliminates additional extraction steps. Ammonium bicarbonate/ammonia buffer at pH 9.7 was used to work at very basic pH. Moreover, ammonium bicarbonate buffer at pH 7.9 has also been selected in order to prevent instability of some compounds at high pH.

For working at a slightly lower pH, ammonium acetate buffer (pH: 6.6) was selected. It is a suitable option for those compounds that can be unstable in acidic or basic conditions. In our experience its use in the analysis of different pharmaceuticals provides good selectivity and excellent peak shapes. Ammonium formate/formic buffer (pH: 3.1) was also selected because in our experience it helps in solving some difficult separations. Moreover, it is often used with HILIC columns [28].

Acid aqueous solutions have also been extensively used in analytical [29] and preparative HPLC [30,31] in the field of medicinal chemistry for the analysis of those compounds with a carboxylic group. In this work formic acid has been selected because it is recommended in HPLC–MS and in addition, it provides a simple way to dramatically change selectivity in HPLC. Ammonium acetate, ammonium formate and formic acid are also appropriate to be used with atmospheric pressure ionization sources due to their volatility.

Other typical HPLC modifiers such as trifluoroacetic acid were not included in this work because salts can be easily formed with the analytes, and moreover an increase in background noise occurred. However, it is an option to be considered if none of the solvents and buffers tested provides enough selectivity.

3.3. Columns selection

Several columns of different characteristics, three reversed phase C-18 columns, a phenyl column and an HILIC silica column were selected. The XBridge C-18 column was the first to be chosen because of its high stability working at high pH and the "universal" selectivity provided by the C-18 functionality. It must be commented that, in our experience, with these columns the best results in terms of lifetime and efficiency are always obtained if they are used at the conditions for which they have been specifically designed. For instance, for C-18 columns working at neutral and acidic conditions, the Sunfire C-18 column operates better than the XBridge one because Sunfire Prep™ silica particles have a higher surface area (see Table 2). In this work, the Atlantis dC-18 was also selected to take advantage of its higher retention capabilities,

Table 2
Physical characteristics of the columns.

Column	Pore size (Å)	Surface area (m^2/g)	% Carbon load	Hydrophobicity (*k*, ethylbenzene)[a]	Silanol activity (*k* /Tailing factor, amitriptyline)[a]
XBridge C-18	135	185	17.5	1.63	3.79/1.10
SunFire C-18	100	340	16	2.5	6.3/1.1
Atlantis C-18	100	330	14	1.7	5.1/2.4
XBridge Phenyl	157	187	15.06	–	–/1.21
HILIC	100	330	14	–	–

[a] Silanol activity and hydrophobicity values taken from the US Pharmacopeia LC "L1" (C18) column listing.

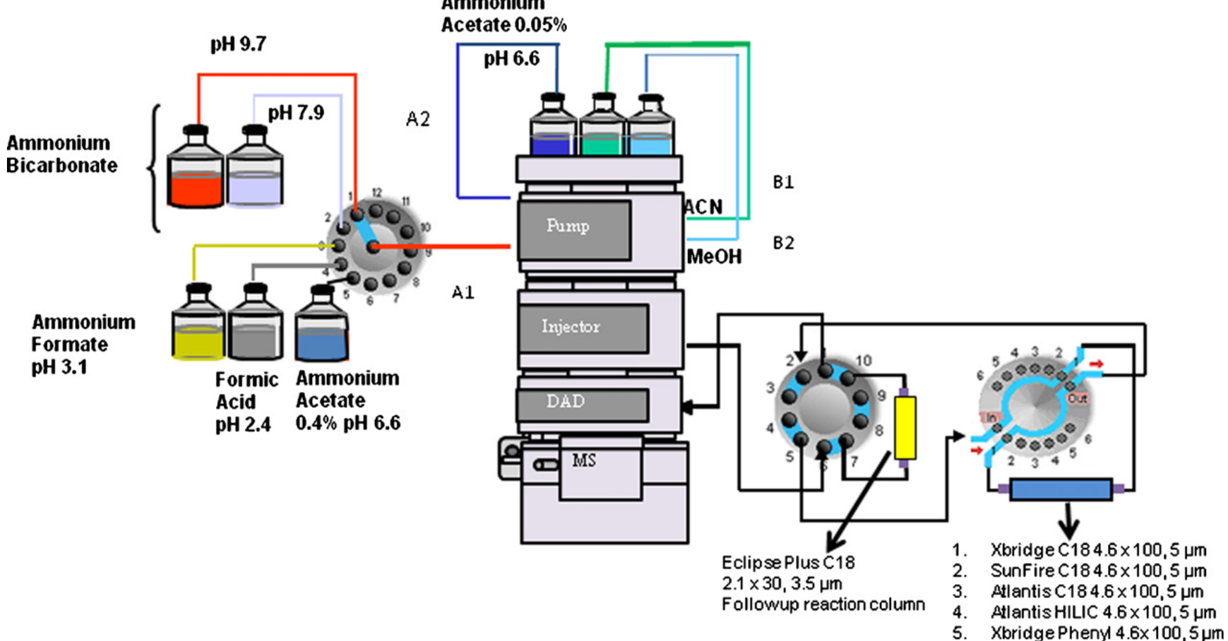

Fig. 1. HPLC–MS configuration for orthogonal HPLC method development. Several column stationary phases and buffers can be easily selected automatically.

compared to the other stationary phases used in this study. The stationary phase attributes of this column such as endcapping, silica pore size, bonded phase ligand density and ligand type yields a higher retention for polar analytes and also exhibits a different selectivity compared to the other C-18 phases used. Table 2 shows some physical characteristics of these three columns that can explain the different selectivity observed.

Another column that has been included in the study is a phenyl one, the XBridge Phenyl, which was selected to take advantage of its different selectivity particularly because of the enhanced π–π interactions with aromatic molecules. Again the Xbridge technology allowed working at a high pH. In addition, we have used hydrophilic interaction chromatography (HILIC) which is an orthogonal method to reversed phase HPLC that offers a different retention mechanism [32]. It is a variation of normal phase chromatography that uses a polar (hydrophilic) stationary phase and a mobile phase with a high percentage of organic solvent (95–80%) and a low proportion of aqueous phase. An Atlantis HILIC silica column was selected with the main objective of retaining polar compounds that cannot be retained using reversed phase columns.

All the analytical columns used in this study had their equivalent preparative "Optimum Bed Density" (OBD) columns. These special packings results in preparative columns with much greater efficiency and a longer lifetime than those packed with conventional techniques. In order to have an easy translation from the analytical method to the preparative one, all the columns selected for the screening had the same particle size and length than the preparative ones that are going to be used in the purification laboratory.

A diagram of all the methods that can be the performed in the system built are given in Fig. 2.

3.4. System reproducibility

One of the difficulties found in the set up was the reproducibility of the analysis, as we dramatically changed conditions from one analysis to the next one. To solve this problem we chose to

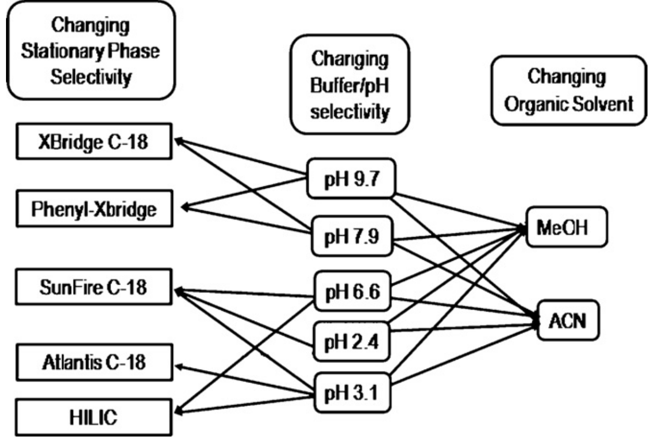

Fig. 2. Diagram showing all the possible methods that can be run in our screening device.

Table 3
Retention times and retention times (n:4) relative standard deviations (%RSD).

Conditions	Caffeine (1)	p-Nitrophenol (2)	Chlorthalidone (3)	Hydroxicumarine (4)	Haloperidol (5)	Flavone (6)	Nabumetone (7)
XBridge C-18 pH 9.7 ACN	2.6/0.08	1.8/0.4	3.5/0.03	3.2/0.01	5.6/0.05	5.5/0.06	5.7/0.06
XBridge C-18 pH 9.7 MeOH	3.7/0.04	2.2/0.07	4.7/0.04	4.5/0.04	6.7/0.04	6.6/0.03	6.5/0.1
XBridge C-18 pH 7.9 ACN	2.6/0.06	2.7/0.8	3.6/0.03	3.3/0.06	5.5/0.03	5.5/0.03	5.7/0.03
XBridge C-18 pH 7.9 MeOH	3.7/0.02	3.3/0.08	4.8/0.04	4.6/0.03	6.5/0.02	6.6/0.02	6.6/0.02
SunFire C-18 pH 2.4 ACN	2.8/0.02	4.5/0.04	3.8/0.02	5.7/0.03	3.4/0.08	5.8/0.03	6.1/0.03
SunFire C-18 pH 2.4 MeOH	4.0/0.04	5.5/0.1	4.8/0.03	6.7/0.02	4.7/0.2	6.9/0.03	6.8/0.1
SunFire C-18 pH 3.1 ACN	2.8/0.3	4.4/0.2	3.7/0.7	5.6/0.5	3.7/0.2	5.8/0.4	6.0/0.5
SunFire C-18 pH 3.1 MeOH	4.1/0.2	5.5/0.1	4.8/0.03	6.7/0.03	5.1/0.2	6.9/0.05	6.8/0.03
Atlantis C-18 pH 3.1 ACN	3.1/0.1	4.6/0.04	4.0/0.04	5.7/0.01	4.5/0.2	6.0/0.01	6.1/0.4
Atlantis C-18 pH 3.1 MeOH	4.4/0.1	5.4/0.07	4.9/0.06	6.8/0.07	5.7/0.09	7.0/0.07	6.9/0.06
XBridge Phenyl, pH 9.7 ACN	2.8/0.02	1.9/0.3	3.6/0.04	3.1/0.07	5.6/0.02	5.2/0.01	5.5/0.01
XBridge Phenyl, pH 9.7 MeOH	4.4/0.1	2.3/0.04	4.8/0.04	4.6/0.04	6.9/0.01	6.7/0.01	6.6/0.01
XBridge Phenyl, pH 7.9 ACN	2.8/0.4	2.7/0.1	3.7/0.02	3.2/0.02	6.1/0.04	5.2/0.04	5.5/0.04
XBridge Phenyl, pH 7.9 MeOH	3.1/0.1	4.6/0.04	4.0/0.04	5.7/0.01	4.5/0.2	5.9/0.02	6.1/0.02
SunFire C-18 pH 6.6 ACN	2.8/0.04	4.3/0.2	3.8/0.06	4.1/0.8	4.7/0.7	5.8/0.04	6.1/0.04
SunFire C-18 pH 6.6 MeOH	4.1/0.7	5.3/0.6	4.8/0.5	5.7/0.4	6.2/1.2	6.9/0.5	6.8/0.5
HILIC pH 6.6 ACN	1.0/0.03	0.7/0.02	0.7/0.02	0.4/0.2	3.5/0.1	0.7/0.02	0.7/0.02
HILIC pH 6.6 MeOH	0.8/0.01	0.6/0.2	0.7/0.03	0.4/0.6	1.5/0.6	0.7/0.03	0.7/0.03
HILIC pH 3.1 ACN	1.0/0.1	0.7/0.04	0.6/0.01	0.6/0.01	2.9/0.04	0.7/0.04	0.6/0.01
HILIC pH 3.1 MeOH	0.8/0.01	0.7/0.03	0.7/0.03	0.7/0.03	0.9/0.3	0.7/0.03	0.7/0.03

inject a blank sample between one condition and the following one. This methodology worked well for all the columns and conditions except for the Sunfire C18 column with ammonium acetate. In this case we could not obtain a good reproducibility in the chromatograms probably due to the specific sequence applied only to this column which involved a big pH difference (3.1–6.6) when changing from ammonium formate to ammonium acetate buffer. For this reason we had to use two SunFire C-18 columns, one for acidic conditions and the other one for ammonium acetate. To check the reproducibility of the chromatographic system we run four times our standard mixture in the whole screening protocol. The relative standard deviations (%RSD) for the retention times in the standard mixture for four consecutive injections in a screening sequence are given in Table 3. A high reproducibility in retention times with %RSD values between 0.01 and 1.2 for the compounds of the standard mixture was achieved.

3.5. Sample introduction and data treatment

The Agilent's Chemstation used in this work provided an easy way of introducing sample names and methods for both individual samples and a sequence. Agilent's Easy Access software is a tool developed to make it easy for both experienced and non-experienced users, to program the analysis of samples in an open access environment. However, for programming a whole set of experiments for one sample or for several ones, it was needed

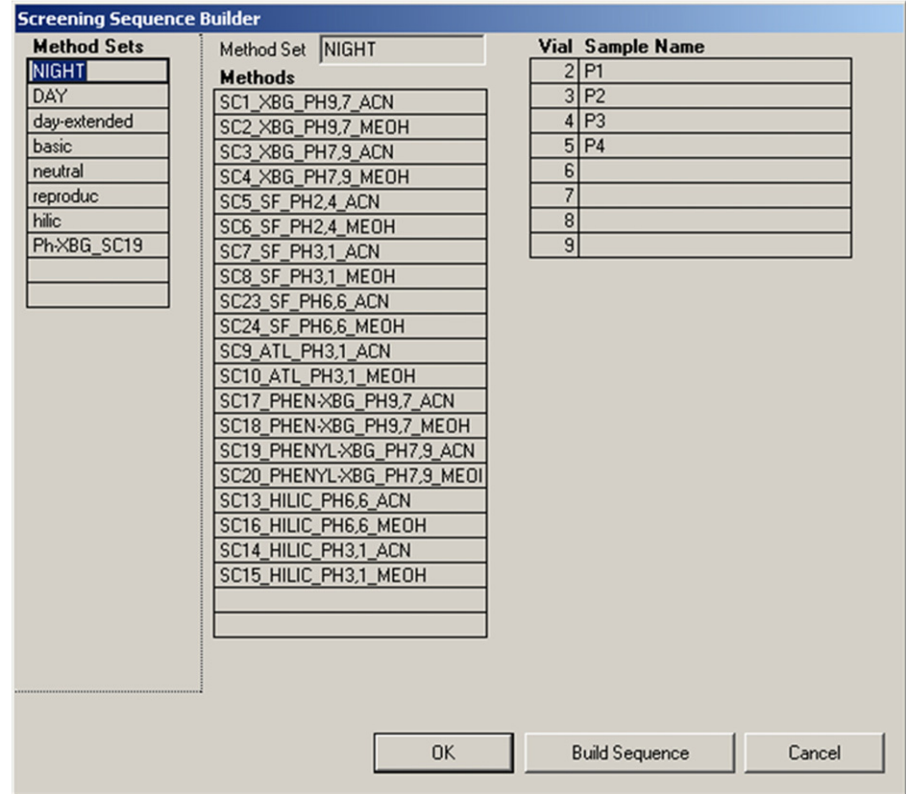

Fig. 3. View of the Screening Builder method programming page.

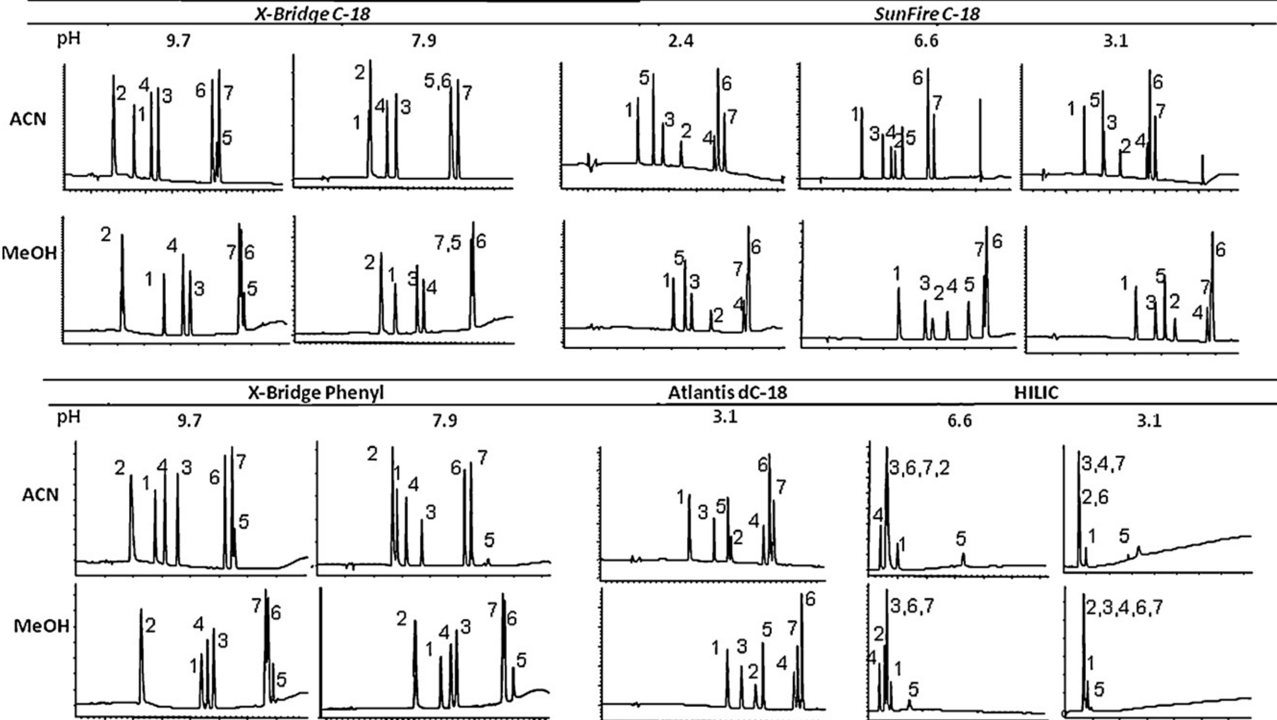

Fig. 4. Chromatograms of the standard mixture under different columns and conditions. Compounds have been identified in the chromatogram by the molecular ion reported by the screening builder (not shown). 1: caffeine, 2: p-nitrophenol, 3: chlorthalidone, 4: hydroxicumarine, 5: haloperidol, 6: flavone, 7: nabumetone.

additional software development. For this purpose, a new software tool was developed in collaboration with Leoson (Middelburg, The Netherlands). The new tool called Screening Builder allowed to easily program a list of the desired runs in order to find the best method. In Fig. 3 a view of the screen of the method programming is shown.

With this software the whole set of experiments or only some of them can be selected taking into account the properties of the compounds of interest. For example, if a compound has a low p*K*a only the set of experiments that use low pH conditions are selected. This new tool also includes a blank run before each new method to stabilize the column for the next run.

As the number of experiments registered is quite high, there is a need for a customized report with all the information in an easy format. A report with all the UV chromatograms acquired, including relative area purities (%), Rt and the base peak mass detected in positive mode for all the peaks is elaborated automatically by the Screening Builder, so results can be reviewed within a few seconds and a decision can be easily made. The information of the base peak mass is of great help to identify the compound of interest in the mixture and to confirm the mass spectrometry data needed for the mass triggered purification.

3.6. Separations of the compounds of the standard mixture

To study the capability of the developed system a mixture of several pharmaceutical compounds (Table 1) was analyzed. The compounds were selected taking into account their different p*K*a, functionalities and retention times along the chromatogram. The chromatograms obtained using different columns, pHs and organic solvents are depicted in Fig. 4. As can be observed the chromatograms are quite different indicating that the conditions selected covered a broad selectivity range that would be enough to solve different separation problems. For instance, when using the column XBridge C-18 at pH 9.7, the simple change from methanol to acetonitrile clearly changes the profile of the peaks allowing separating those with numbers 5, 6 and 7. Lowering the pH to 7.9 the retention time of peak number 2 (p-nitrophenol), increased, changing the elution order of this compound and peak 1 (caffeine) and decreasing their resolution. Moreover, at this pH the separation of the three last eluting compounds worsened, compounds 5 and 6 coeluted with ACN and 5 and 7 with methanol. Another relevant change in selectivity can be observed when changing from pH 2.4 to 3.1 with the SunFire C-18 column making difficult the separation of chlortalidone (peak 3) from haloperidol (peak 5) with ACN.

As mentioned above Atlantis dC-18 is not only useful to increase the retention of early eluting peaks but also because it presents different selectivity. This can be observed in the chromatograms obtained for the standard mixture at pH 3.1 in the Sunfire C-18 and in the Atlantis dC-18 columns. Retention of peak number 5 (haloperidol) increased in the Atlantis column thus improving the separation between this compound and chlorthalidone (peak 3). Higher retention for some compounds also occurred when using the column XBridge–Phenyl instead of XBridge C-18 at pH 9.7 for instance the increase of the retention of peak 4 due to the contribution of the π–π interactions with aromatic molecules can explain the change of resolution between peaks 1, 4 and 3. The HILIC column provided short retention times for most of the compounds, even for the most retained in the other columns and conditions although generally coelutions occurred.

3.7. Applicability to synthesis mixtures

As commented previously, most of the crude mixtures generated in our synthesis laboratories are purified by normal phase chromatography or by our standard starting preparative chromatography conditions. It is not uncommon that after these procedures some of the samples have close eluting impurities that should be eliminated before the pharmacological studies.

The advantage of using the method development proposed in this work is shown in the next examples where we have achieved

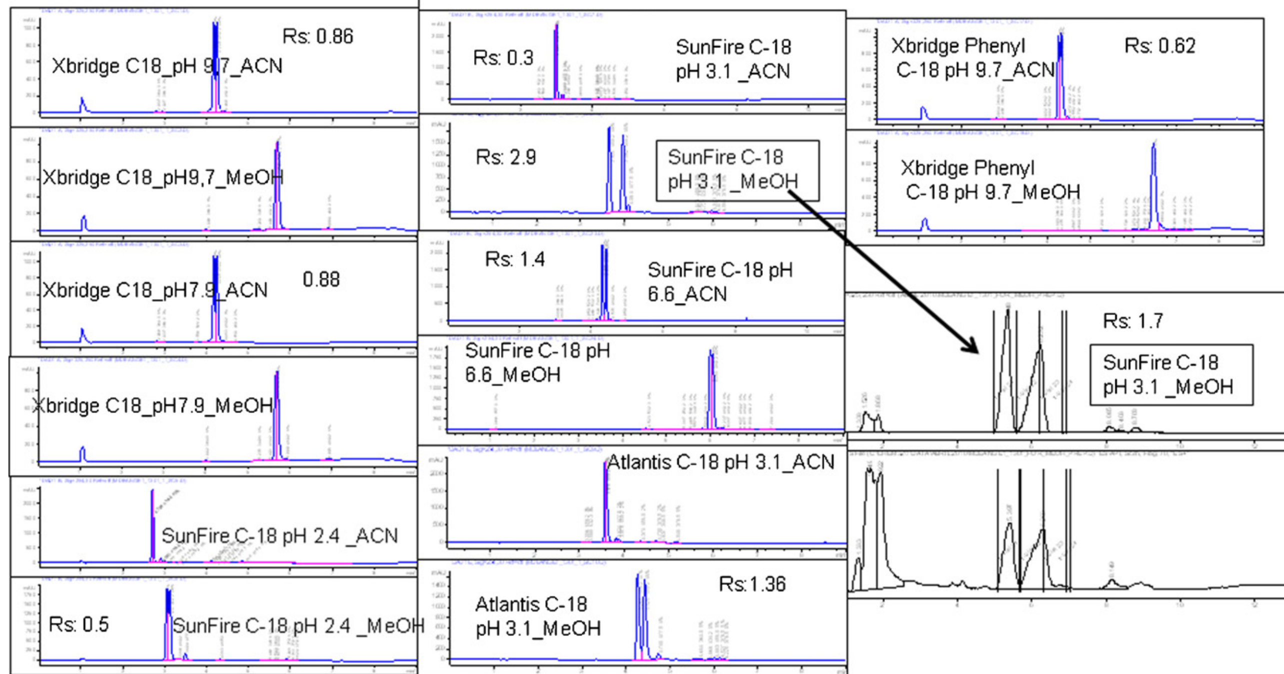

Fig. 5. Results of the analytical screening of a crude mixture from synthesis. In those chromatograms some separation is observed between the two main peaks, the 18 resolution is shown. The analysis with SunFire C-18 and formic acid/ACN, gave the better resolution (Rs: 2.9). The preparative chromatography of 50 mg of the mixture was done with SunFire C18 100 × 19 5 μm ammonium formate/ACN, 20 mL/min from 80/20 to 100% of organic solvent. Preparative chromatogram shows the DAD, the total ion chromatogram and the collection marks.

the purification of critical pairs of compounds. Chromatograms of Fig. 5 corresponded to the analysis of a mixture obtained in the laboratory of synthesis containing the expected compound but also a regioisomer. With the method of screening proposed in this work we could find several separation conditions, in this case a column Sunfire C-18 and a mobile phase with ammonium formate/MeOH, led us to separate the compound and the impurity (Fig. 5). These conditions were translated to the equivalent preparative conditions yielding the desired pure compound to the chemist. At the end of the process the desired compound was obtained with a 97% of purity checked by UPLC–MS and NMR.

Another example is included in Fig. 6 where the separation of a synthesis mixture containing a minor impurity is shown. Three different conditions provided the separation between the expected

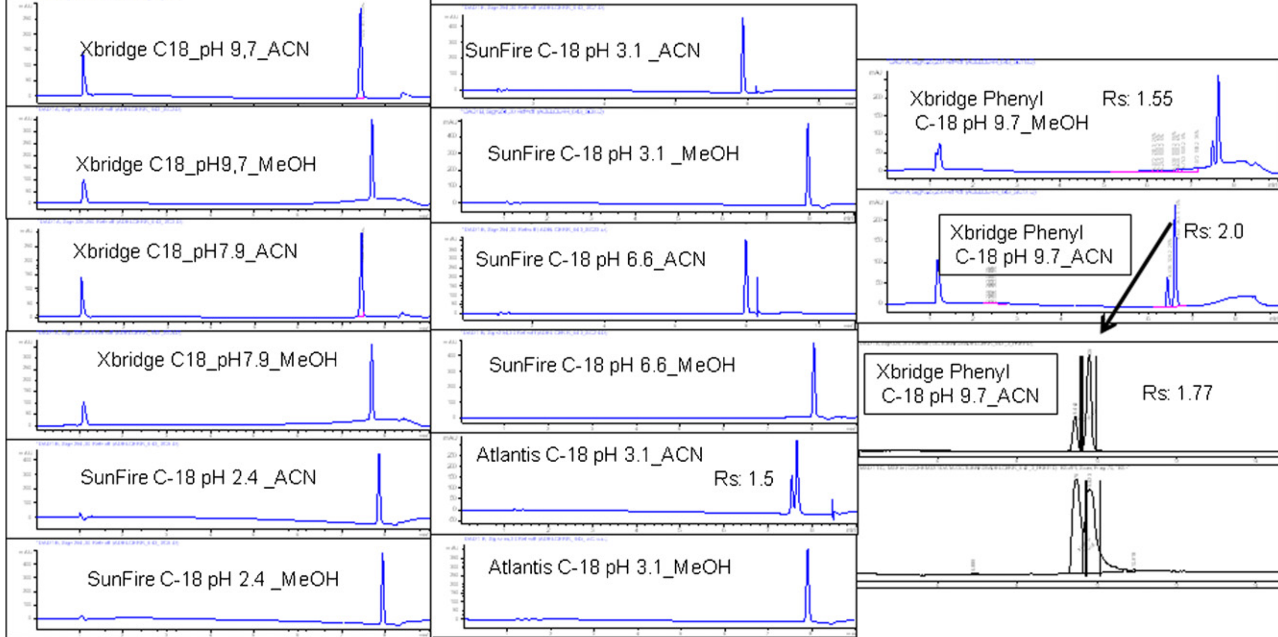

Fig. 6. Results of the analytical screening of a crude mixture from synthesis. In those chromatograms were some separation is observed between the two main peaks, the resolution is shown. XBridge Phenyl ammonium bicarbonate-NH_4OH/ACN, gave the better resolution (Rs: 2.0). The preparative chromatography of 75 mg of the mixture was done with XBridge Phenyl 100 × 19 5 μm ammonium bicarbonate + NH_4OH/ACN, 20 mL/min from 80/20 to 100% of organic solvent. Preparative chromatogram shows the DAD, the total ion chromatogram and the collection marks.

compound and the main impurity. The best resolution (2.0) was obtained with ammonium bicarbonate-NH_4OH pH 9.7/ACN in a XBridge Phenyl column. The desired compound was recovered from the corresponding preparative column with a 100% of purity, checked by UPLC–MS and NMR.

4. Conclusions

In the present paper we have developed an analytical screening procedure to be used for preparative separations, by modifying one of our open-access liquid chromatography–mass spectrometry instruments. The modification of one of our analytical "open access" HPLC–MS systems to perform the analytical screening while maintaining the open-access functionality for synthesis reaction monitoring, the searching methodology and the use of the mass spectrometry information obtained from the screening for the identification and triggering of the fraction collection are all novel aspects of this work.

The searching methodology proposed herein covers a wide range of HPLC conditions providing a general method useful for the separation and purification of different kinds of compound mixtures. We have demonstrated through the use of standards and mixtures from synthesis procedures the utility of the device and of the methodology proposed. As we are using the same particle size and length in the analytical columns and in the preparative columns it is quite straightforward to perform the method translation. Screening Builder, the software tool developed for the method of screening, improves the automation not only in the input of sample data but also in the report output which allows fast interpretation of results. Using Screening Builder with a pre-set list of methods, expert and non-expert users can obtain valuable information about the best purification method to be chosen.

Acknowledgements

We would like to thank Jose Manuel Alonso for his support in NMR validation of compounds obtained from the HPLC–MS preparative purifications, Fernando Resano for his help in running samples in the analytical screening device and Dr. Gary Tresadern for the text corrections.

References

[1] H.N. Weller, M.G. Young, S.J. Michalczyk, G.H. Reitnauer, R.S. Cooley, P.C. Rahn, D.J. Loyd, D. Fiore, S.J. Fischman, Mol. Divers. 3 (1997) 61.
[2] L. Zeng, L. Burton, K. Yung, B. Shushan, D.B. Kassel, J. Chromatogr. A 794 (1998) 3.
[3] P.J. Edwards, Comb. Chem. High Throughput Screen. 6 (2003) 11.
[4] C.R. Johnson, B. Zhang, P. Fantauzzi, M. Hocker, K.M. Yager, Tetrahedrom 54 (1998) 4097.
[5] U.J. Nilsson, J. Chromatogr. A 885 (2000) 305.
[6] A. Espada, M. Molina-Martin, J. Dage, M.-S. Kuo, Drug Discov. Today 13 (2008) 417.
[7] A. Weber, E. von Roedern, H.U. Srilz, J. Comb. Chem. 7 (2005) 178.
[8] G.M. Rishton, Med. Chem. 1 (2005) 519.
[9] O. Guth, D. Krewer, B. Freudenberg, C. Paulitz, M. Hauser, K. Ilg, J. Comb. Chem. 10 (2008) 875.
[10] J. Pellet, P. Lukulay, Y. Mao, W. Bowen, R. Reed, M. Ma, R.C. Munger, J.W. Dolan, L. Wrisley, K. Medwid, N.P. Totl, C.C. Chan, M. Skibic, K. Biswas, K.A. Wells, L.R. Snyder, J. Chromatogr. A 1101 (2006) 122.
[11] L.R. Snyder, J. Chromatogr. B 689 (1997) 105.
[12] N.S. Wilson, M.D. Nelson, J.W. Dolan, L.R. Snyder, P.W. Carr, J. Chromatogr. A 961 (2002) 195.
[13] M. Molina-Martin, A. Marin, A. Rivera-Sagredo, A. Espada, J. Sep. Sci. 28 (2005) 1742.
[14] X. Gang, A.D. Bendick, R. Chen, S.S. Sekulic, J. Chromatogr. A 1050 (2004) 159–171.
[15] K.M. Biswas, B.C. Castle, B.A. Olsen, D.S. Risley, M.J. Skibic, P.B. Wright, J. Pharm. Biomed. Anal. 49 (2009) 692.
[16] A. Marín, K. Burton, A. Rivera-Sagredo, A. Espada, C. Byrne, C. White, G. Sharman, L. Goodwin, J. Liq. Chromatogr. Related Technol. 31 (2008) 2.
[17] E. Loeser, S. Babiak, P. Zhu, G. Yowell, M. Konigsberger, P. Drumm, Chromatographia 63 (2006) 345.
[18] E.F. Hewitt, P. Lulukay, S. Galushko, J. Chromatogr. A. 1107 (2006) 79.
[19] E. Francotte, H.A. Wetli, J. Sep. Sci. 30 (2007) 1255.
[20] L. Zeng, R. Xu, D.B. Laskar, D.B. Kassel, J. Chromatogr. A. 1169 (2007) 193.
[21] A. Espada, A. Marin, C. Anta, J. Chromatogr. A. 1030 (2004) 43.
[22] K.F. Blom, J. Comb. Chem. 4 (2002) 295.
[23] K.F. Blom, B. Glass, R. Sparks, A.P. Combs, J. Comb. Chem. 6 (2004), 874 -.
[24] L.M. Font, A. Fontana, M.T. Galceran, L. Iturrino, V. Perez, Orthogonal analytical screening for HPLC method development and preparative scale-up. Poster presented at HPLC 2009 held in Dresden-Germany.
[25] L.M. Font, A. Fontana, M.T. Galceran, L. Iturrino, J. Oyarzabal, HPLC–MS purification of compounds using p*K*a prediction for method selection. Poster presented at HPLC 2005 held in Stockholm-Sweden.
[26] A. Espada, A. Rivera-Sagredo, J. Chromatogr. A. 987 (2003) 211.
[27] A. Espada, A. Marín, C. Anta, J. Chromatogr. A. 1030 (2004) 43.
[28] X. Zhang, X. Zhao, C. Zhang, L. Yang, X. Xiong, Y. Zhou, Y. Yang, J. Duan, Chromatographia 71 (2010) 1015.
[29] D.A. Yurek, D.L. Branch, M.-S. Kuo, J. Comb. Chem 4 (2002) 138.
[30] M. Bauser, J. Chromatogr. Sci. 40 (2002) 292.
[31] W. Leister, K. Strauss, D. Wisnoski, Z. Zhao, C. Lindsley, J. Comb. Chem. 5 (2003) 322.
[32] X. Wang, L. Weiyong, H.T. Rasmussen, J. Chromatogr. A. 1083 (2005) 58.