Screening ionisation and chromatography conditions for quantitative LC/MS methods

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ABSTRACT

To develop an optimal quantitative LC/MS method with high sensitivity, high selectivity and robustness in a limited time period can be very challenging, especially for methods in which many analytes are to be quantified. In this study the relevant options are reviewed and a simple screening strategy of mass spectrometric and chromatographic conditions is presented. The strategy is divided into two stages, mass spectrometric ionisation screening and reversed phase LC column screening. The objective of the first stage is to find out how sensitivity is affected by ionisation technique, ionisation polarity and buffer. The compounds are dissolved in different buffers covering a broad pH range. Thereafter they are injected using flow injection analysis without LC column, evaluating both electrospray and atmospheric pressure chemical ionisation (APCI). In the second stage the buffers yielding the best sensitivity and selectivity in the ionisation screening stage are used as mobile phase buffers to LC column screening with different stationary phases applying a shallow gradient. The aim is to find the combinations of column(s) and buffer(s) that give symmetric peaks, adequate retention and selectivity. Finally the retention is adjusted using isocratic or gradient elution. The strategy provides a simple and practical experimental design that allows fast screening a large range of ionisation and chromatographic conditions especially for multiple compounds. The examples included in this study demonstrate that optimal buffer, ionisation technique, ionisation polarity and column cannot be predicted from compound properties such as structure and pK_a.

1. Introduction

The introduction of easy to use atmospheric pressure ionisation interfaces for mass spectrometry has been a revolution for liquid chromatography. Many scientists found it easy to obtain qualitative information allowing for elucidation and/or confirmation of molecular structures. It was soon realised that LC/MS could also be used for quantitative analysis with a sensitivity that was usually better than e.g. ultraviolet detection. Furthermore, LC/MS offered a far better selectivity and, in most cases, reducing need for chromatographic resolution. LC/MS is now an established technique for quantification of low concentrations of analytes in complex matrices.

During the first era of this technique it was perceived that method development could be reduced or even eliminated. Consequently many users have reduced method development to a minimum and generic methods are frequently used, especially in the pharmaceutical industry. When drugs and related substances

are to be determined in *in vitro* or *in vivo* samples generic methods are common practice within early drug screening stage. When a drug compound is selected for development the generic method may not meet demands on e.g. selectivity, robustness and sensitivity needed for *in vivo* samples [1–7]. Yet, the time allowed for method development is still limited. Modifications of sample preparation procedures often have a major impact on method performance but consume significant method development time. Changing ionisation and/or chromatography conditions can be a faster route to improve methods. This may thus be the main (or even only) part of method development. It is therefore essential that this work can be done efficiently.

This article reviews relevant options for ionisation and chromatography. A time efficient and simple strategy for screening of ionisation and chromatography suited for the practitioner is presented. The strategy aims for bioanalysis in later parts of drug development but may well be applied in other areas.

The strategy (Fig. 1) is easy to implement without specific hardware or software requirements. The aim is to find ionisation and chromatography method conditions that give high sensitivity and selectivity, high chromatographic resolution and short cycle times. In addition, more than one set of conditions is identified for each

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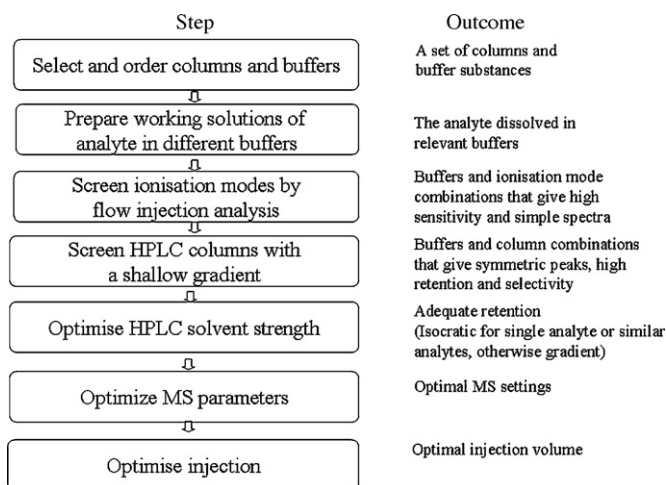[1] Previously Agilent Technologies, Kista, Sweden.

Fig. 1. Flow chart description of method development strategy.

analyte to provide alternatives when e.g. matching with sample preparation.

2. Experimental

2.1. Chemicals and reagents

The following chemicals and solvents were used: acetonitrile (HPLC grade, Rathburn), methanol (HPLC grade, Rathburn), formic acid (pro analysis, Merck), acetic acid (glacial, J.T. Baker), ammonium formate (puriss p.a., Fluka), ammonium acetate (puriss p.a., Fluka), trifluoroacetic acid (for protein sequencing analysis, Sigma–Aldrich), ammonium hydrogencarbonate (ultra, Fluka), ammonia 25% solution (pro analysis, Merck), water (Elga quality, Elga), 1-(cyclohexylamino)-3-[(2-methyl-1H-indol-4-yl)oxy]-2-propanol (Bionet), SUC-PHE-GLY-LEU-BETA-NA (Nova Biochem, peptide analogue), 2-(1-amantyl)quinoline-4-carboxylic acid (Maybridge), (S)-di-naphthylprolinol (Aldrich), 2-benzylamino-1,1-diphenyl-1-ethanol (Bionet), pindolol (Sigma), 2-[(4-chlorobenzyl)amino]-1,1-diphenyl-1-ethanol (Bionet), testosterone (Sigma–Aldrich), caffeine (Sigma–Aldrich), theophylline (Fluka). Structures are given in Fig. 2.

2.2. Instrumentation

Chromatographic separation and mass spectrometric measurements were carried out either on an Agilent 1100 liquid chromatography system (Agilent Technologies, Waldbronn, Germany) equipped with solvent degasser, low or high pressure mixing pump (low pressure for column screening and high pressure for gradient elution optimisation), wellplate autosampler, diode array detector and a quadropole mass spectrometer (G1946D), or a Shimadzu liquid chromatography system (Shimadzu, Tokyo, Japan) equipped with solvent degasser, gradient pump (LC-10AD), autosampler (SIL-HT), and an API4000 triple quadropole mass spectrometer (MDS Sciex, Canada).

Acquisition was done both in positive and negative mode using both electrospray (turbo ion spray) and APCI ionisation modes.

The columns used were Waters Symmetry® C$_{18}$, Thermo Hypersil Gold C$_{18}$, Waters Xbridge™ Shield RP18, Phenomenex Gemini C^{18} 110 Å and Thermo HyPurity C$_{18}$. They were all 3 mm × 50 mm and packed with 3–3.5 μm particles.
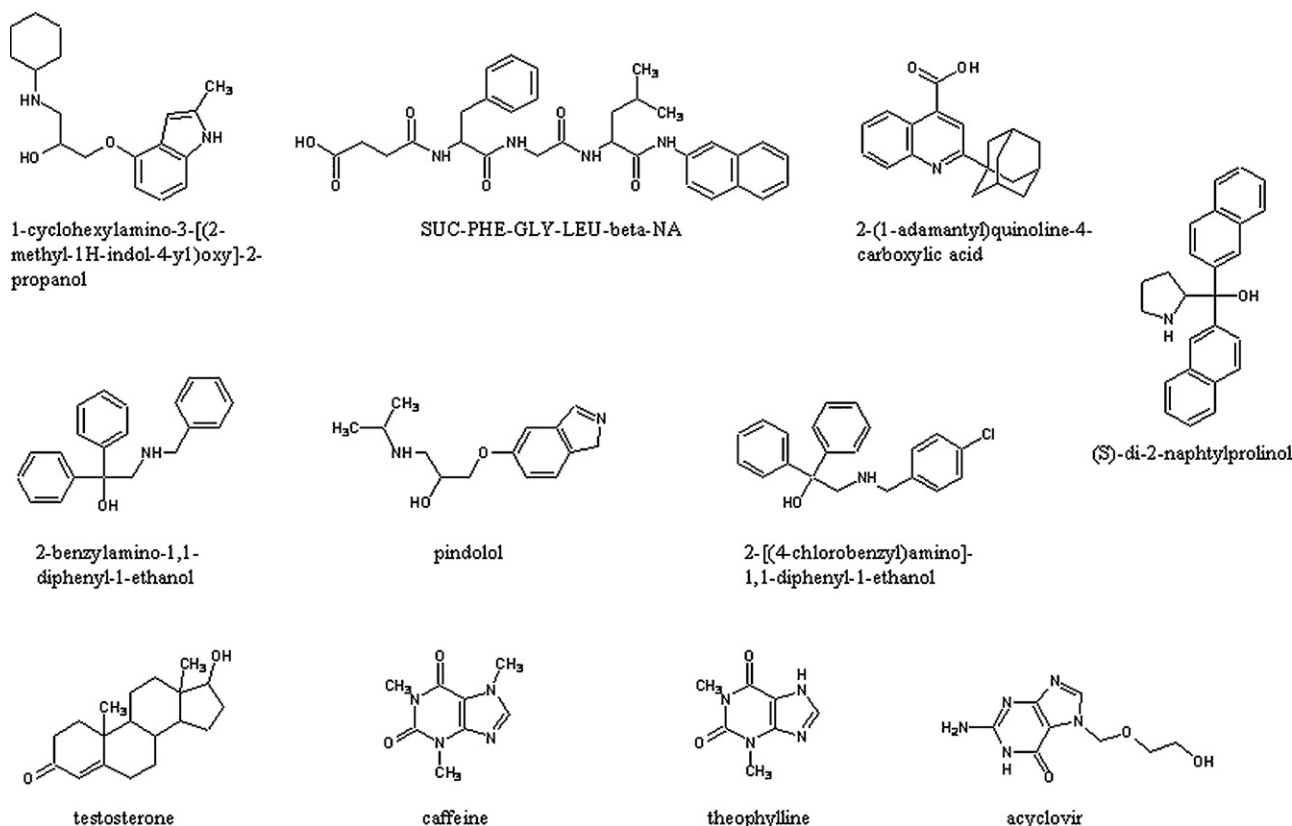

Fig. 2. Structures of compounds used.

Table 1
Buffers evaluated in this work.

pH	Buffer and pH	Preparation
2.0	10 mM TFA	770 μL of TFA ⇒ 1 L
3.0	20 mM formic acid	760 μL of 99% formic acid ⇒ 1 L
4.0	5 mM formic acid + 10 mM ammonium formate	190 μL of 99% formic acid, 0.64 g of ammonium formate ⇒ 1 L
5.0	5 mM acetic acid + 10 mM ammonium acetate	280 μL of 99% Acetic acid, 0.77 g of ammonium acetate ⇒ 1 L
∼7	10 mM ammonium acetate	0.77 g of ammonium acetate ⇒ 1 L
8.1	10 mM ammonium hydrogencarbonate	0.80 g of ammonium hydrogencarbonate ⇒ 1 L
8.9	10 mM ammoniumacetate + 5 mM ammonia	770 mg of ammonium acetate + 400 μl 25% ammonia ⇒ 1 L
11	10 mM ammonia	800 μL 25% ammonia → 1 L

2.3. Solutions

Stock buffer solutions of the eight different buffer components were prepared in water to a concentration of 200 mM trifluoroacetic acid, 400 mM formic acid, 100 mM formic acid with 200 mM ammonium formate (pH 3.9), 100 mM acetic acid with 200 mM ammonium acetate (pH 5.0), 200 mM ammonium acetate (native pH 6), 200 mM ammonium hydrogencarbonate (native pH 8), 200 mM ammonium acetate with 100 mM ammonia (pH 9.4) and 200 mM ammonia.

The test compounds to be used were dissolved either in acetonitrile, acetonitrile–water mixture or 10 mM formic acid in acetonitrile–water mixtures to obtain stock solutions with a concentration of 50 μM.

2.4. Flow injection conditions for the ionisation screening

The chromatographic system was set up with a mobile phase of acetonitrile and water (50:50). The flow was set at 0.5 mL/min and no column was installed in the system. Mass spectrometric parameters were set to default according to the recommendations from the instrument vendors. The compounds were diluted to a concentration of 2–3 μM in each of the eight different buffers given in Table 1 and acetonitrile (50:50) in one vial per buffer. A reference vial with the compound dissolved in only water and acetonitrile was also used. Injections of samples were done in automated sequences.

2.5. Chromatographic conditions for the column screening and elution optimisation

During column screening the gradients were run with an increase of 5% acetonitrile per minute at a flow rate of 1 mL/min. The starting concentration of acetonitrile was adjusted for each substance to allow elution with the same gradient profile for different buffers and columns.

Retention optimisation for isocratic elution was done with a flow rate of 0.6 mL/min. The concentration of acetonitrile was changed in steps of 2% between each run. An equilibration time of 4 min was allowed before each injection.

Retention optimisation for gradient elution was done with a flow rate of 1.0 mL/min. The gradients were run with an increase of 10% of acetonitrile per minute. The starting concentration of acetonitrile was changed in steps of 5% between each run. An equilibration time of 4 min was allowed before each injection.

In all experiments the columns were thermostated at 40 °C. Stock solutions of test compounds were diluted in a mixture of acetonitrile and water (no buffer) and injected in volumes of 1–5 μL. Detection was made by positive electrospray.

3. Results and discussion

3.1. Ionisation screening

The first stage in the strategy is ionisation screening. The objective is to find the ionisation technique and ionisation mode, precursor ion(s) and product ion(s), and preferred buffers (mobile phases) with the best sensitivity and selectivity which will be used in the succeeding column screening. In addition, it is also an efficient way to achieve an overview of how the compounds of interest behave in different ionisation conditions. This is e.g. useful for LC/MS methods covering multiple analytes for which optimal ionisation conditions have to be a compromise.

In the ionisation screening the different buffers are injected together with the compound to be optimised instead of being added to the mobile phase, which is usually the standard approach. This allows for a faster evaluation of different buffers.

The HPLC system was set up with water and acetonitrile without addition of buffer or column. The system flow rate was 0.5 mL/min. The compounds were diluted from the stock solutions in the different buffers with the same organic solvent and content as on the HPLC system to an analyte concentration of 2–3 μM. The analysis was done with Flow Injection Analysis, FIA, using large injections of 30–50 μL with both electrospray and APCI in both positive and negative mode. In this work the ionisation polarities were acquired in separate experiments. By injecting rather large volumes without using a column, it is ensured that when the compounds reach the ion source, they will be present in the buffer in which they are dissolved and that the obtained result reflects the effect of each specific buffer. The instrument parameters on the mass spectrometer were set to default and the acquisition was done by scanning over a broad range, e.g. $m/z = 70 - [2Mw + 100]$. A qualitative evaluation of the spectra in each peak is done to find out which ions are formed and if they vary with pH, buffer, organic solvent (if tested) and ionisation technique.

An example is the peptide seen in Fig. 3 where the most abundant adduct ions, i.e. $m/z = 561$ [M+H]$^+$ and $m/z = 583$ [M+Na]$^+$, and also fragment ions, vary with the selected buffer. In this example the different fragments seen are an indication that different product ions might form depending on precursor ion used in MS/MS. The most promising ions, based on sensitivity, selectivity, etc., to be used as a precursor are extracted. Thereafter the buffers yielding the highest peaks are selected. Ideally it will result in at least two different sets of mass spectrometric conditions that show high sensitivity and selectivity. In this example positive electrospray using [M+H]$^+$ with ammonium hydrogen carbonate (Fig. 3a and b) or negative APCI using [M−H]-(Fig. 3c and d) with ammonium hydrogen carbonate or ammonia as buffer.

Another example is 2-benzylamino-1,1-diphenyl-1-ethanol, for which either positive electrospray (Fig. 4a and b) using formic acid or ammonium hydrogencarbonate or APCI using any of the buffers could be used (Fig. 4c).

A summary of the relative base peak intensities obtained using the two interfaces and each of the different buffers for the compounds tested are shown in Table 2. It should be noted that only intensities (and not signal to noise) are evaluated. Comparison of intensities is believed to be sufficient for screening purposes with a predefined set of buffers with known properties and a selective detector (if LC/MS, and not LC/MS/MS, is the technique to be used a more thorough evaluation of signal to noise might be needed).
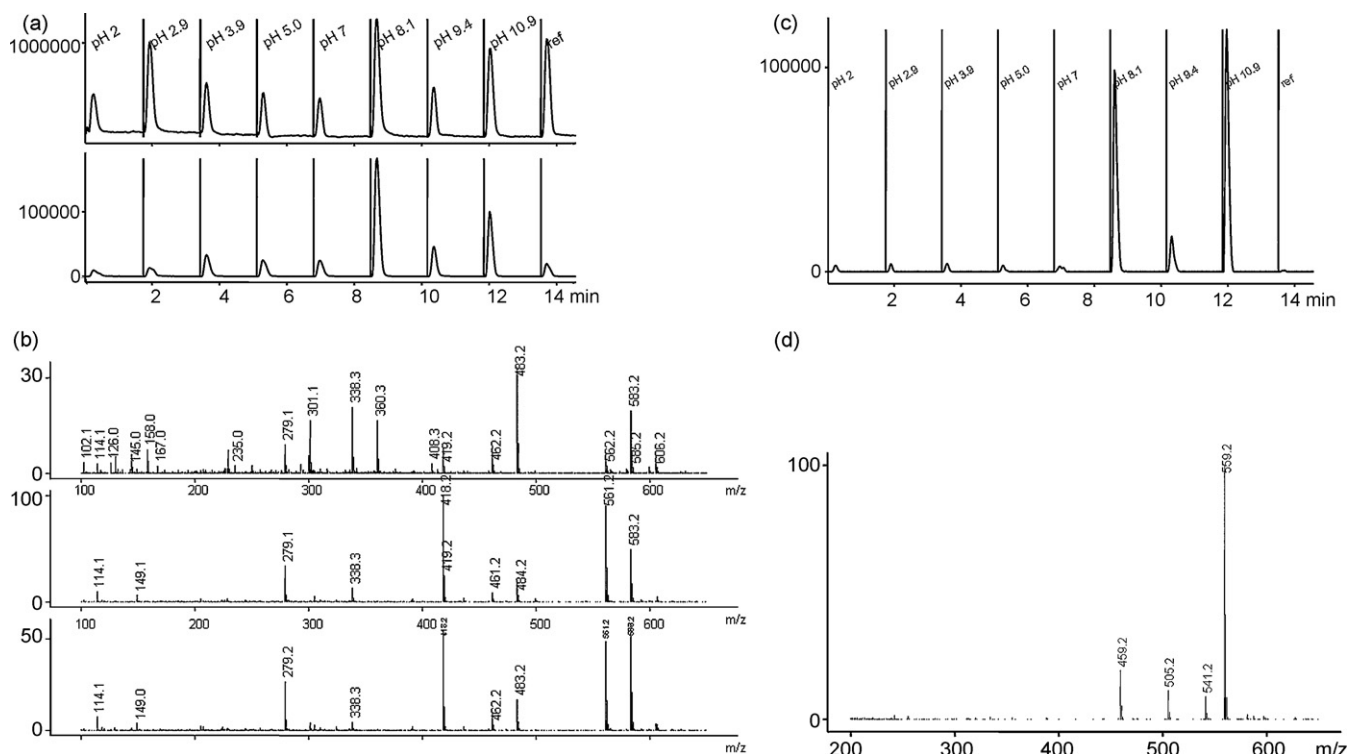
Fig. 3. Ionisation screening of the peptide SUC-PHE-GLY-LEU-BETA-NA using (a) positive electrospray. TIC (top) and EIC of m/z 561 (bottom). (b) Positive electrospray spectra acquired in formic acid (top) ammonium hydrogencarbonate (middle) and ammonia (bottom). (c) Negative APCI EIC of m/z 559. (d) Negative APCI spectrum acquired in ammonium hydrogencarbonate.

If the method is used with tandem mass spectrometry, the precursor sensitivity and selectivity monitored so far are not the only critical factors for overall sensitivity and selectivity. In addition, the efficiency of fragmentation of the precursors into product ions is as important. Therefore, to be able to select the best buffer it might be necessary to continue with MS/MS. Typically this can be done rather

quickly without tedious optimisation, simply by comparing product ions formed and the fragmentation efficiencies of the potential precursor ions.

In this work both electrospray and APCI were evaluated. APPI, the third common commercially available interface, may be included in the strategy. It should also be mentioned that the
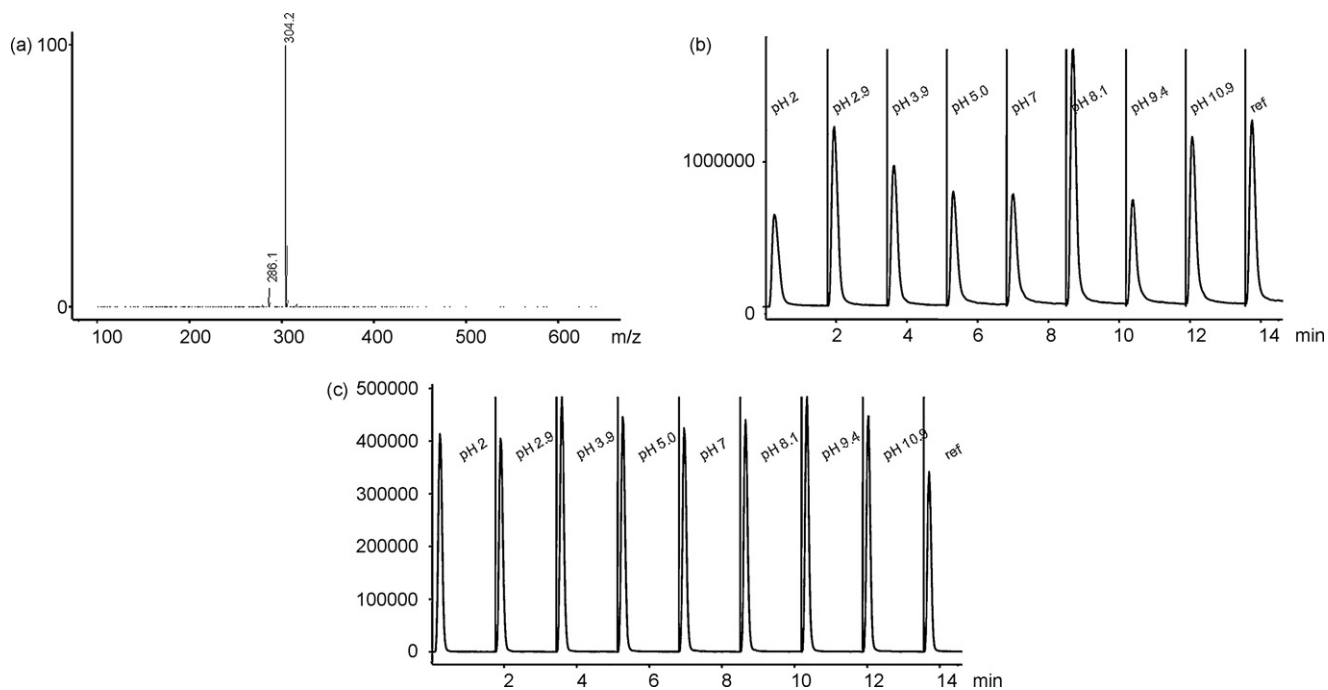

Fig. 4. Ionisation screening of 2-benzylamino-1,1-diphenyl-1-ethanol. (a) Positive electrospray spectrum from compound dissolved ammonium hydrogen carbonate. The spectra were similar for all buffers and also for positive APCI and therefore not shown. (b) FIA of positive electrospray. EIC of m/z 304. (c) FIA of positive APCI. EIC of m/z 304.

Table 2

Summary of the relative base peak intensities of the compounds using the four different ionisation modes in ionisation screening on the Agilent single quadropole instrument. 100 correspond to the highest relative intensity of the compound regardless of ionisation technique. The base peak ion in the different buffers is the same for one compound, but might be different for positive versus negative and electrospray versus APCI, respectively. The base peak ion is typically the protonated or deprotonated molecule.

Compound	Ionisation	Relative intensities (%)							
		pH 2.0	pH 3.0	pH 4.0	pH 5.0	pH 7	pH 8.1	pH 8.9	pH 11
1-(Cyclohexylamino)-3-[(2-methyl-1H-indol-4-yl)oxy]-2-propanol	ESI +	5	48	49	22	20	100	40	46
	ESI –	–	–	–	–	–	–	–	–
	APCI +	39	37	41	38	20	20	25	22
	APCI –	–	–	–	–	–	–	–	–
SUC-PHE-GLY-LEU-BETA-NA	ESI +	8	5	19	15	17	100	17	29
	ESI –	9	23	13	16	21	42	25	47
	APCI +	2	1	1	1	3	13	10	20
	APCI –	3	2	2	1	8	54	24	61
2-(1-Adamantyl)quinoline-4-carboxylic acid	ESI +	16	28	30	10	12	100	21	43
	ESI –	–	1	2	2	2	5	2	5
	APCI +	15	12	13	10	10	13	8	9
	APCI –	0	7	8	6	6	9	7	9
(S)-Di-2-naphtylprolinol	ESI +	34	69	59	47	49	100	45	70
	ESI –	–	–	–	–	–	–	–	–
	APCI +	20	15	33	22	21	19	19	17
	APCI –	–	–	–	–	–	–	–	–
2-(Benzylamino)-1,1-dipheyl-1-ethanol	ESI +	36	69	54	43	43	100	40	53
	ESI –	–	–	–	–	–	–	–	–
	APCI +	36	34	42	39	40	42	43	42
	APCI –	–	–	–	–	–	–	–	–
Pindolol	ESI +	31	48	42	28	33	100	33	60
	ESI –	–	–	–	–	–	–	–	–
	APCI +	25	21	26	25	25	25	21	25
	APCI –	–	–	–	–	–	–	–	–
2-[(4-Chlorobenzyl)amino]1,1-diphenyl-1-ethanol	ESI +	32	83	79	47	50	100	53	50
	ESI –	–	2	4	6	6	2	5	5
	APCI +	55	60	63	66	65	66	68	64
	APCI –	0	1	1	2	2	2	2	2
Testosterone	ESI +	9	13	33	18	21	100	23	39
	ESI –	–	–	–	–	–	–	–	–
	APCI +	65	73	24	22	22	21	14	19
	APCI –	–	–	–	–	–	–	–	–
Caffeine	ESI +	6	7	38	12	13	78	16	23
	ESI –	–	–	–	–	–	–	–	–
	APCI +	100	91	71	72	68	64	69	69
	APCI –	–	–	–	–	–	–	–	–
Theophylline	ESI +	4	4	16	5	5	88	4	26
	ESI –	0	45	38	53	49	49	23	75
	APCI +	44	40	4	4	4	4	4	4
	APCI –	0	35	43	39	39	58	45	52
Theophylline in methanol instead of acetonitrile[a]	ESI +	6	7	16	6	7	15	9	15
	ESI –	NA	NA	NA	NA	NA	NA	NA	NA
	APCI +	95	69	30	48	36	100	26	40
	APCI –	0	18	22	25	15	14	30	16

(–) Not ionised to a detectable level; NA, not applicable.

[a] Relative intensities are for theophylline in both acetonitrile and methanol.

outcome of the ionisation screening might be instrument vendor specific [8].

3.1.1. Comparison of ionisation techniques

To be able to do the ionisation screening efficiently it is vital that the default ion source and acquisition parameters on the mass spectrometer can be used. In contrast to early ion source designs, modern types of interfaces need little optimisation [9]. During evaluation one also has to consider that while electrospray (and APPI) tends to require a limited ion source optimisation beyond default parameters to achieve the optimal sensitivity, APCI results can change significantly when changing these parameters. Therefore, when comparing the results of electrospray and APCI acquired with default settings one must keep in mind that it is easy to underestimate the APCI result. For applications where default APCI

parameter settings give similar result considering sensitivity as electrospray or in situations when electrospray sensitivity is not sufficient the APCI parameters should be optimised.

For the example given in Fig. 4 the sensitivity of positive electrospray with ammonium hydrogencarbonate is about twice as high compared to positive APCI with any of the buffers. This is a typical example where APCI should be evaluated further to optimise the sensitivity. It should also be noted that some of the newer ion sources use simultaneous electrospray and APCI, electrospray and APPI or APCI and APPI [10]. Usage of such interface could further simplify the procedure for the ionisation screening.

There are more or less established rules for which compounds ionise well on electrospray versus APCI [11,12], but this work shows that these rules may be misleading. For example, even though it is known that small acidic peptides, like SUC-PHE-GLY-LEU-BETA-

NA, might ionise in negative mode and also in APCI these conditions are not the most common choice for peptides (Table 2). Also caffeine that would have been expected to ionise best with electrospray, ionised comparably well using APCI. The most commonly used ionisation technique is currently electrospray. This is probably due to its simplicity and easy optimisation. In the authors' experience many compounds ionise with both techniques and which one is the best is difficult to predict. Furthermore, APCI tends to result in more linear relations between concentration and response and generally is less influenced by matrix effects than electrospray [13–15], which would motivate a broader use of this technique in quantitative applications.

As can be seen for the peptide, the background generated by the different interfaces varies. Generally the background is highest for electrospray followed by APCI and lowest for APPI. Depending on the application this may be an important factor that has to be taken into consideration when evaluating the ionisation screening.

For many compounds the formation of multiple adducts is common. This is more frequent for electrospray, while it is less frequent for APCI and APPI. There are several publications of the impact and occurrence of adducts, how to reduce or increase the abundance of them [16–19]. In the proposed strategy it is suggested to select ionisation and buffer combinations yielding as simple spectra as possible as long as the sensitivity is as good as or better than conditions where more adducts are formed. The use of ionisation screening with multiple buffers and ionisation modes provides the spectral information needed to make this selection. There will inevitably be situations where multiple adducts cannot be avoided. For those situations precaution has to be taken during validation of the methods. The peptide, SUC-PHE-GLY-LEU-BETA-NA, seen in Fig. 3 forms [M+H]$^+$ and [M+Na]$^+$ among a number of adduct ions. Working with complex biological samples, the concentration of sodium might vary, and be different from calibration samples. Therefore, if possible, it is advisable to select conditions minimising the sodium adduct and promoting the proton adduct. Extraction of the [M+H]$^+$ ions clearly demonstrates that ammonium hydrogencarbonate, followed by ammonia, will result in the highest abundance of [M+H]$^+$.

3.1.2. Comparison of buffers

It has previously been pointed out that the selection of mobile phase affects both the mass spectrometric result as well as chromatographic behaviour of the analyte at hand [20]. The range of buffers evaluated in this work is given in Table 1.

As expected, electrospray sensitivity was more affected by pH and buffer additive compared with APCI. However, it was found that with very few exceptions that pH was not the most critical parameter for electrospray sensitivity. Instead the choice of buffer component was most important. This is supported by the fact that ammonium hydrogencarbonate (pH 8) gave high a sensitivity for most of the compounds using positive electrospray. Buffers containing acetate usually resulted in the lowest sensitivity. This finding could explain why so called "wrong way around" ionisation, ionisation at a pH where the molecule is uncharged [21,22] works well for many applications.

The differences in response between the buffers can also be due to the different buffer concentrations and the result would probably be different if these concentrations were changed [23]. However, the buffer concentration will be a compromise between buffer capacity and ionisation efficiency. It was also noticed that the effect of the buffers using the ionisation screening varied when using different instruments.

3.1.3. Comparison of organic solvents for mobile phase

The selection of organic solvents suitable as mobile phase is more limited than for buffers. The primary candidate is acetoni-

trile and the second one usually methanol. The higher viscosity of methanol makes it less attractive, but there have been reports of significant differences in mass spectrometric response and adduct formation between these solvents [20,24,25] and even between different brands of methanol [26]. For the compounds tested in this screening only theophylline showed significantly different results in methanol compared with acetonitrile. Another interesting fact is that the relative effect of the different buffers may be different if methanol is chosen instead of acetonitrile, as seen for theophylline (see Table 2).

There are occasions reported where better sensitivity can be achieved using other solvents than acetonitrile and methanol [10,15] especially if APCI is the ionisation technique chosen.

3.2. Column screening

When the ionisation screening has been completed a small set of buffers for mobile phase remains to be used in the column screening stage. In this stage the aim is to identify reversed phase columns that give symmetric peaks, high retention and adequate separation if there are multiple analytes.

The selection of column length, diameter, flow rate, temperature and particle size is all interconnected [27]. In this work columns were selected that can be used with ordinary HPLC system with cycle times of less than 3 min for isocratic and 7 min for gradient elution. From the stated requirements a column length of 50 mm with a diameter of 3 mm and a packing material of 3–3.5 μm was selected. The column temperature was set at 40 °C and the flow rate was 1.0 mL/min for gradients and 0.6 mL/min for isocratic elution.

The selection of stationary phases to include in screening is more difficult systematic characterisation of stationary phases using a well defined generic test would provide data to compare and select stationary phases based on identical data. However, no general agreed evaluation procedure is used by column manufacturers, instead they present their columns with data that are difficult to compare. Public databases of column characteristics established by independent organisations using generic test are becoming available and will hopefully mature to support an objective selection of stationary phases.

In this work a set of C$_{18}$ columns has been chosen as they have high retention and stability. Columns that have high retention will give appropriate retention times using mobile phases with high proportions of organic solvent. The latter will often improve sensitivity as the ionisation efficiency usually increases with the percentage of organic solvent. Furthermore, larger sample volumes may be injected as column focusing increases in comparison with columns that give less retention.

The column screening is done by gradient elution as this allows columns to be compared without modification of elution conditions. The mobile phases are made with the buffers found optimal during ionisation screening. The gradient should be fairly flat (<3% change in organic solvent/column volume) to show differences in peak shape, retention and selectivity.

An example of the outcome of column screening for one selected compound is given in Tables 3 and 4.

Table 3
Retention times (min) for 2-benzylamino-1,1-diphenyl-1-ethanol with different mobile phase buffers and columns. Gradient elution as described in experimental.

pH	Gold	Symmetry	Gemini	Xbridge	HyPurity
2	4.4				
3	4.2	2.7	3.2	2.7	4.1
4	4.3	4.0	4.3	3.9	4.3
8	8.6		9.5	8.7	
11			9.7		

Table 4
Peak symmetry for 2-benzylamino-1,1-diphenyl-1-ethanol with different mobile phase buffers and columns. Symmetry as calculated by Chemstation (1.0 is highest possible symmetry), Gradient elution as described in experimental.

pH	Gold	Symmetry	Gemini	Xbridge	HyPurity
2	0.9				
3	0.6	0.9	0.5	0.9	0.9
4	0.9	0.8	0.7	1.0	0.9
8	1.0		0.8	0.9	
11			0.8		
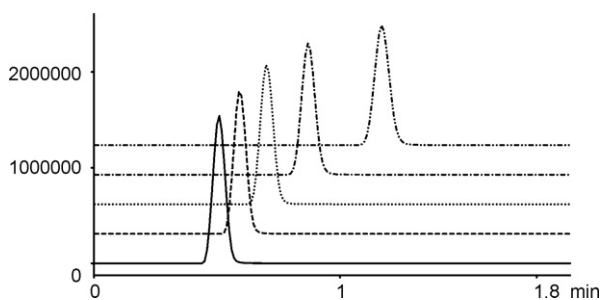
Fig. 5. Optimising retention with isocratic elution by varying amount of acetonitrile in steps of 2%.

3.3. Retention optimisation

In the last optimisation step the retention is adjusted by changing the amount of organic solvent. The initial decision is selection of isocratic or gradient elution. Retention optimisation for isocratic elution is done by repeated injections while lowering the concentration of organic solvent. The aim is to have a k' of at least 3. An example is shown in Fig. 5.

Retention optimisation for gradient elution is done by having a fixed gradient slope of 4%/column volume. This gives approximately an average capacity factor of five for small molecules and is thus a good compromise between run time and resolution [28]. The starting concentration is lowered until the retention time is approximately four times the dead time. Examples of gradient optimisation are given in Fig. 6.

There are several reliable commercial software's that can reduce the number of experiments that need to be done in this step [29]. Yet, the short run times in this work enables retention optimisation to be done in less than 1 h and thus allow possible time saving by using an optimisation software small.

3.4. Injection volume

Sensitivity is often a major objective when developing an LC/MS method. A simple way of increasing sensitivity is to inject larger sample volumes. In Figs. 7 and 8 the injection volume is increased
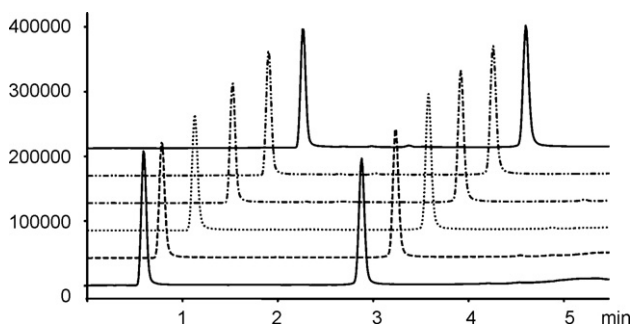

Fig. 6. Optimising retention with gradient elution by varying starting concentration of acetonitrile in steps of 5%.
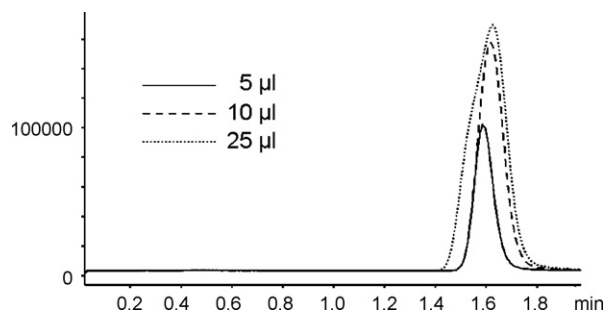

Fig. 7. Injection of increasing volume of sample (5, 10, and 25 μL) with a mobile phase of pH 3 and 24% acetonitrile. Sample dissolved in 24% acetonitrile.
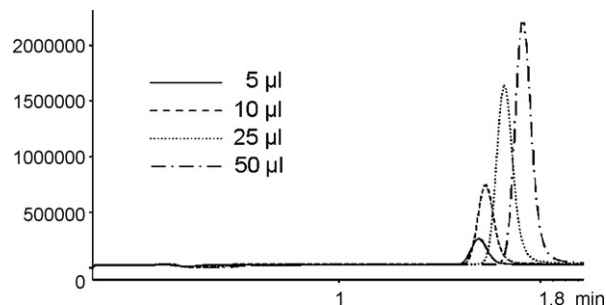

Fig. 8. Injection of increasing volume of sample (5, 10, 25, and 50 μL) with a mobile phase of pH 8 and 62% acetonitrile. Sample solvent as in Fig. 7.

Table 5
Estimated experimental times.

Step	Estimated experimental time	Prerequisites	Comments
Preparation working solutions of analytes	2 h	Solubility	Time may be reduced significantly if stock solutions of buffer are already prepared
Ionisation screening	2 h	Template methods	Several analytes can be done in parallel
Column screening	0.5 h/buffer and column	Template methods	
Elution optimisation	1 h		

with two different mobile phase buffers where the percentage of acetonitrile has been adjusted to obtain equal retention. It is apparent that fairly large volumes may be injected if the sample solvent is weaker than the mobile phase. Finding a buffer and column combination that is highly retentive is thus beneficial for sensitivity.

4. Summary and conclusions

The time allocated for method development during drug development is limited. A generic sample preparation procedure is often used. High sensitivity and selectivity is vital for LC/MS to minimise sample load on column and interferences.

This work hopefully provides a simple and practical experimental design that allows screening of a large range of ionisation and chromatographic conditions within a few hours for multiple compounds in parallel (Table 5).

The examples provided show that the best choice of buffer and ionisation technique is not always as expected from compound properties. Optimal conditions are therefore difficult to predict and common rules may be misleading. It also seems that the choice of buffer additive is more important for the sensitivity than the actual

pH. In most cases the outcome of the proposed screening strategy described is more than one set of LC/MS conditions that meet the requirements. This is valuable as it gives alternatives in cases where the conditions first selected fails at a later stage, e.g. when combined with a sample preparation technique or if unexpected matrix effects occur.

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