UNIVERSITAT JAUME·I

Retention time index in LC-MS

Master's Degree in Applied Chromatographic Techniques

Course: 2014/2015

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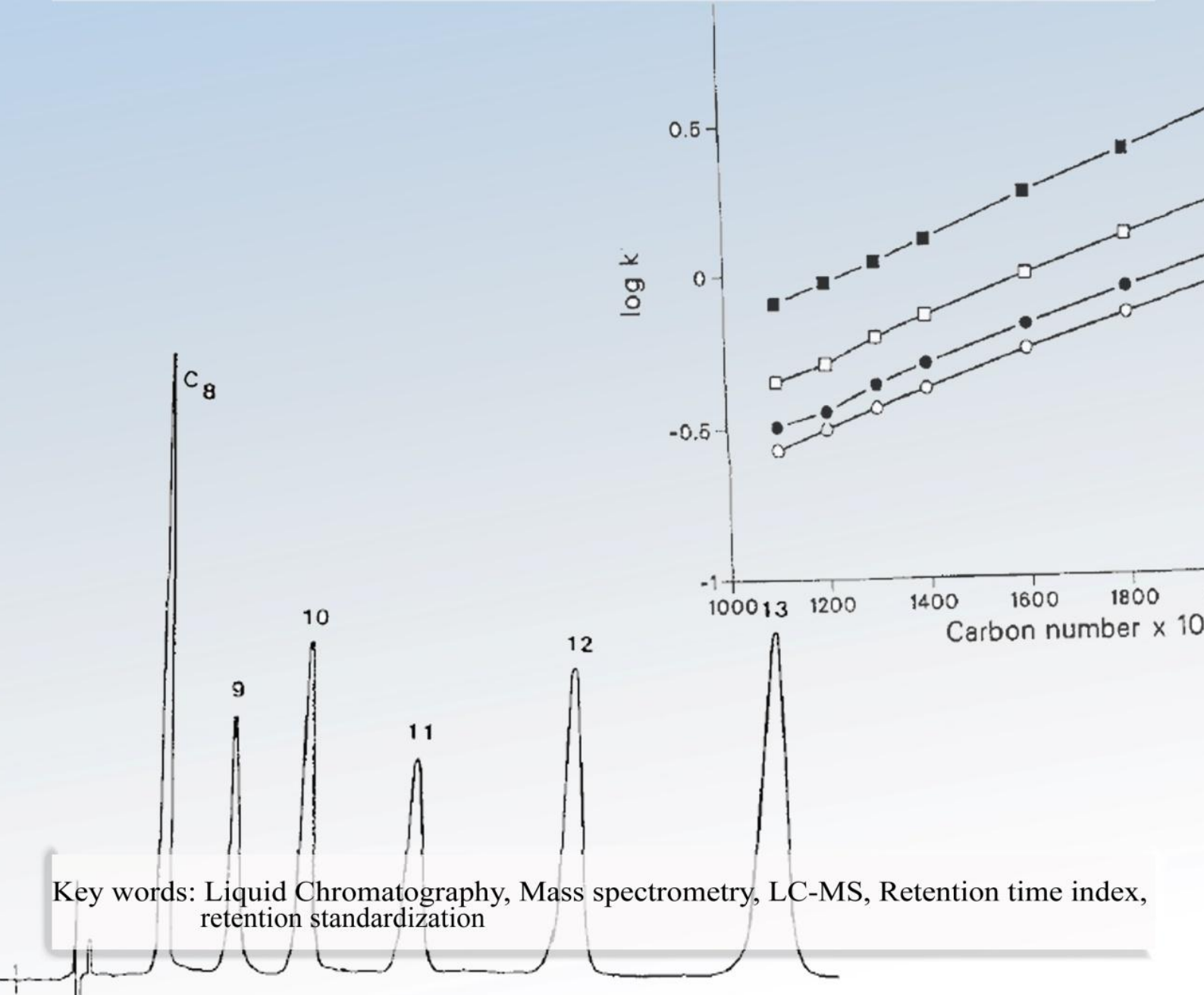
30th of June 2015



Key words: Liquid Chromatography, Mass spectrometry, LC-MS, Retention time index, retention standardization

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1. Introduction

INTRODUCTION TO LC-MS

In the analytical field, one of the separation techniques most used worldwide are liquid (LC) and gas chromatography (GC). This interest comes from the capacity of chromatography to separate complex mixtures in simpler fractions than the initial mix or in separate compounds, making easier the posterior identification of by other techniques. In chromatography, the movement of an analyte through the system is described by its distribution between the stationary and mobile phase (distribution coefficient, K). This movement depends on the physico-chemical properties of the analyte and mobile phase as well as, temperature but it is independent of the amount of analyte in the mixture. So K is characteristic to each analyte and determines the time taken for an analyte to be eluted from a column, called retention time (R_t). Retention time can be used as an identification parameter, especially when spectrophotometric detectors are used [1].

Although LC lacks a universal detector such as flame ionization detector in GC, it is widely used because enables the analysis of thermo labile and highly polar compounds in different matrices as well as the direct injection of liquid samples. The most used LC mode is Reverse Phase Liquid Chromatography (RPLC), where separation is carried out by a non-polar stationary phase placed into the analytical column and a polar mobile phase. In some cases, mobile phases are constituted by different solvents which composition is changing over time (gradient elution). High Performance Liquid Chromatography (HPLC) uses high pressure pumps to push solvents through the column which is shorter than in GC (tipically 5-15 cm) and packed with silica (5-3 μm particle size). To improve chromatographic resolution and achieve a better separation, particle size lower than 2 μm can be used. This type of chromatography works with even higher pressures than HPLC and therefore analysis time becomes shorter. It is referred as Ultra High Performance Liquid Chromatography (UHPLC). *Figure 1* shows the better efficiency at higher linear velocities of UHPLC vs HPLC.

In last decades, interest in coupling UHPLC to mass spectrometry (MS) has increased because of this detector enables lower detection limits and detection based on R_t and mass-to-charge ratio (*m/z*) of a compound. This double identification is very

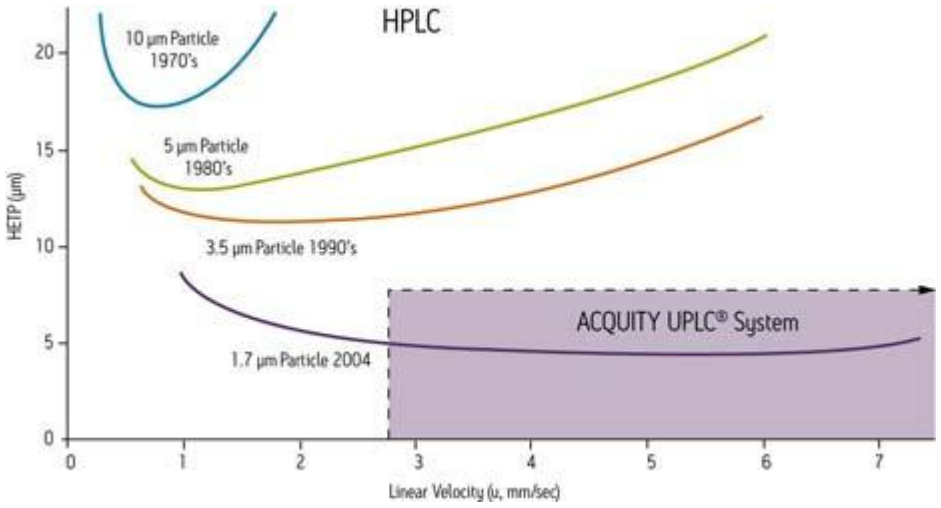

Figure 1. Effect of particle size on resolution and optimum flow rate.

useful as it allows separating compounds which could not be separated by its retention time. Recently, the use of high resolution mass spectrometry (HRMS) has grown, especially in environmental and drug analysis, due to its suitability for both targeted and untargeted analysis. Its main characteristics are the high mass accuracy and sensitivity in full acquisition mode. This makes possible pre- and post- target analysis, retrospective analysis as well as structural elucidation of unknown compounds. It is also interesting to remark the ability to perform screening of a huge number of compounds within one run with high sensitivity [2–5].

Inside the mass spectrometer, ions are generated in the ion source and separated by their mass-to-charge ratio (m/z) in de mass analyzer. Finally, these ions are detected qualitatively and quantitatively by their *m/z* and abundance [2]. A simple scheme of a mass spectrometer is showed in *Figure 2*.

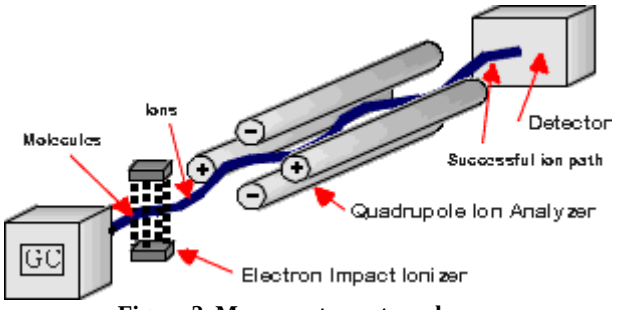

Figure 2. Mass spectrometer scheme.

Nowadays, the most used ionization source is a kind of atmospheric pressure ionization (API) source, named Electrospray ionization source (ESI) (*Figure 3*), developed by John B. Fenn [6]. When ESI interface is used, flow from LC goes through a stainless steel capillary at high potential (3-4 kV to produce positive ions and lightly

lower and of opposite polarity to form negative ions). A concentric gas flow in the capillary together with high potential generate a spray of charged droplets (this process is named nebulization). These droplets are desolvated while they are passing through the atmospheric pressure region of the mass spectrometer source. Finally, the drop breaks due to electrostatic forces are too high, and ions pass to gas phase. Then ions are introduced by the action of a charged cone in the mass analyzer to be separated in the basis of their m/z. *Figure 3* shows a scheme of ion formation in ESI. Several instruments show double orthogonal design with a second cone (extractor) who avoids that non charged substances enter into the mass analyzer. This configuration is known as Z-spray, due to the path that (de)protonated molecules have to follow to enter into the mass spectrometer. *Figure 4* shows the scheme of an electrospray ionization source with Z configuration.

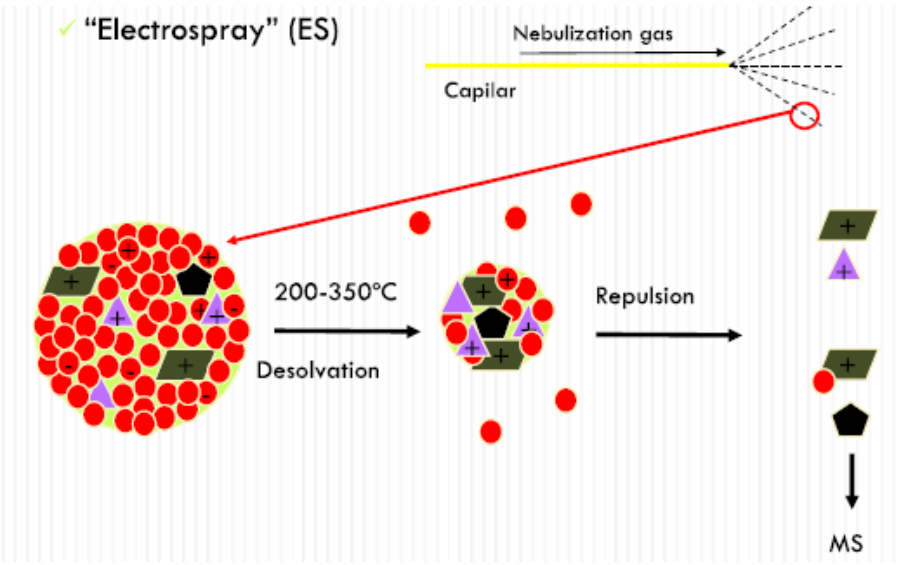

Figure 3. Ionization process.

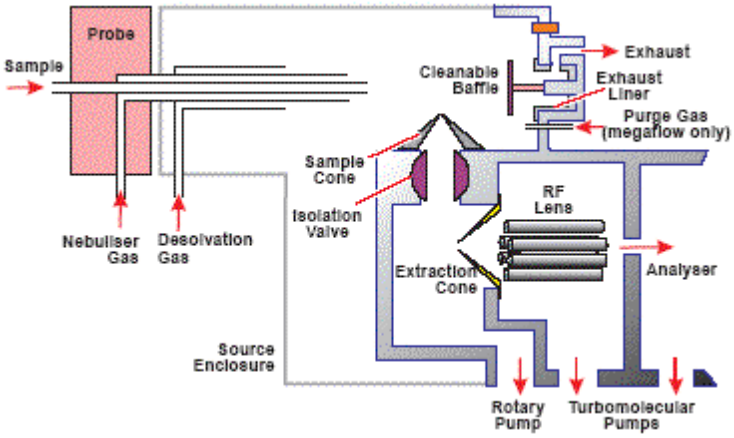

Figure 4. Electrospray ionization source Z-spray design.

In the field of HRMS, different tandem mass configurations have been reported, being one of the most popular quadrupole-time-of-flight (QTOF) [3,7–12]. The operation of TOF analyzer is based on the time taken by an ion to go through a flight tube of known length. The time that takes an ion depends on its m/z relationship. Due to all ions begin its travel at the same time, lighter ions arrive to the detector before than heavier ions. This requires that ions from ion source need to be pushed into the flight tube by an orthogonal pulse from a continuous ion beam. On the fly path, a reflectron corrects the energy dispersion of the ions leaving the source with the same m/z ratio, so ions with the same m/z reach the detector at the same time despite the initial ion dispersion.

When QTOF is working in MS/MS, ions from ion source arrive to the quadrupole, where are filtered and only an *m/z* passes through the quadrupole and arrives to the hexapole collision cell, where this (de)protonated molecule is fragmented and product ions transported into the TOF analyzer. Inside the TOF, fragments are pushed through a flight tube until they reach the detector, recording the time that each fragment has taken to follow the path. Heavier fragments spend more time to reach the detector than lighter fragments (see *Figure 5*).

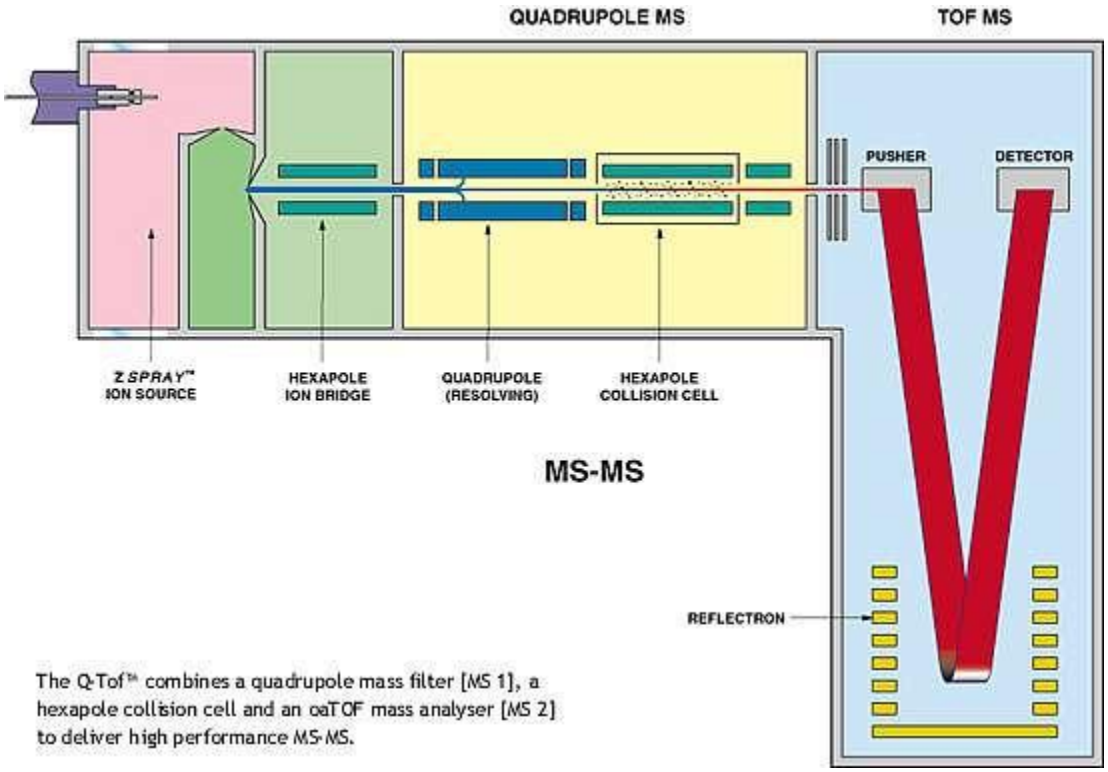

Figure 5. QTOF scheme.

An interesting option for screening purposes is the use of MS^E approach [13–15]. In MS^E experiments, both (de)protonated molecule and fragment ion are simultaneously acquired without previous selection of a precursor. During the acquisition, collision cell switches from low energy (LE) to high energy (HE) in order to generate the molecular information by minimizing fragmentation (LE function) or to produce fragmentation of the molecule (HE), also preserving the isotopic pattern of fragments and dimer and/or adduct information. In this sense, UHPLC becomes very valuable for choosing perfectly co-eluting ions, avoiding interferences that would complicate the identification process. MS^E has been applied to the simultaneous screening and identification of residues and contaminants in different matrices [13,16].

STANDARDIZATION IN LC

Usually, R_t as well as masses of the (de)protonated molecule and fragment ions are included in libraries or target lists to enhance the identification of a compound; however sometimes, retention times are not reproducible in different laboratories, instrumental systems or even using the same instrumental conditions in different days mainly due to analytical column aging or matrix effects. Law et al. [17] demonstrated changes in retention time by using different silica packings for different drugs. Gill et al. [18] performed a study of retention time using the same column packing in different laboratories with distinct instrumentation, and concluded that there were differences in retention time, remarking the need of control of the chromatographic conditions. The change in retention times and selectivity of basic drugs by changes in pH, ionic strength and mobile phase composition was studied by Smith and co-workers [19].

Changes in retention times can cause difficulties in the identification, and even false negative identification could be reported by the analyst. For example, because retention time change from listed value is upper than the retention time tolerance established by SANCO guidelines [20]. As a consequence, different approaches have been used to avoid this problem and a considerable effort has focused in the development of methods of measuring "retention times" and into the development of more reproducible LC systems.

One approach to eliminate variations in R_t is the concept of capacity factors or retention factors (*k*). This approach has been widely adopted in HPLC and lies in the comparison of the retention time of a compound with that of an unretained one. *k* is

defined as the ratio of the adjusted retention time (R_t-R_{tM}) and the retention time of an unretained compound used as a marker (R_{tM}):

$$k = \frac{R_t - R_{tM}}{R_{tM}} \quad (Eq.1)$$

This approach enables to eliminate variations in R_t owing to the flow rate variations and differences in stationary and mobile phases, but faces two problems: the calculation of the void volume (R_{tM}) and the small value of R_{tM} compared with that of an analyte R_t [1].

Another widely adopted approach to improve retention reproducibility in chromatography is the use an internal standard (IS) to report relative retention time as the ratio between the analyte and the IS [21–23]. This method presents some problems, such as the selection of the appropriate IS which has to have similar functional groups with similar pK_a to be affected in a similar manner than the analyte by changes in the chromatographic system. In addition, the selected standard has to be not present in the sample.

HOMOLOGOUS SERIES OF RETENTION STANDARDS

An alternative to use retention times relative to a single IS is to compare the retention of an analyte to a retention index scale of homologous standards. Then, the retention index of the analyte is determined by interpolation of its retention between the retention of the standard compounds.

These homologous series are based in the systematic increase of retention time due to the addition of a methylene group along the homologous series in reversed phase separations. Thus, the retention of a compound (k) in the series can be described by the sum of the retention of a parent compound (k) plus the effect of adding a group to the parent compound (ΔR_M) (Eq.2). This equation was suggested by Martin [24] and means that an homologous series of standard compounds should provide a regularly increasing scale of reference peaks across a chromatographic separation.

$$\log k = \log k_p + \sum \Delta R_M(i) \quad (Eq.2)$$

The increasing of retention due to the addition of a methylene group was applied by Kóvats to develop its retention index scale [25] based in n-alkanes which is widely

applied in GC. N-alkanes are readily available in high purity, chemically stable, inert, non-toxic and their retention times cover a wide range of analyte retentions. As retention time increases with carbon number, the retention indices of the standard is defined as 100 x $n_{C.}$ Retention index for a sample compound is determined by logarithmic interpolation (Eq. 3).

$$I = 100 \cdot \left(\frac{\log t'_{R,i} - log\, t'_{R,z}}{log\, t'_{R,z+1} - log\, t'_{R,z}} \right) + 100z \quad (Eq. 3)$$

Where, $t'_{R,i}$ is the retention time of the analyte, $t'_{R,z}$ is the retention of the next shorter n-alkane and $t'_{R,z+1}$ is the retention time of the next longer n-alkane containing z and z+1 carbon atom. The principal application of the Kóvats indices has been for identification of unknown compounds, although they are also used to compare properties of stationary phases. Zeeuw [26] noted the success of Kóvats retention index scale and the need to develop a similar system in HPLC to standardize the retention time recording. Some studies examined the potential of Kóvats retention index in HPLC but n-alkanes are difficult to detect using spectroscopic detectors and are relatively nonpolar. So they have longer retention than most compounds of interest [27].

In his book, Smith compile criteria to create a widely applicable retention index scale in liquid chromatography [1]:

a) They should have a strong chromophore group at 254 nm so that they can be added to unknown samples in small quantities to act as internal standards. It should be noted that this rule was first formulated by Smith in 1982 [28], where HPLC usually uses spectrophotometric detectors as UV. Working with mass spectrometers, this is not an important feature, due to MS counts ions and not emited or absorbed light by a sample.
b) They should not be readily ionized to avoid changes in retention because of pH variations or the presence of ion-pair reagents. Like in the criterion a, this criterion is only applicable if UV detector will be used. Criterion b forces to exclude any molecule with acidic or basic groups, like OH, NH_2, COOH which could be ionized depending on the pH. These groups are very interesting in MS because they could be easily ionized and detected in the mass spectrometer.

c) A range of members of the series should be readily available at reasonable cost.
d) The most polar member of the series should be eluted with a similar retention to water soluble pharmaceuticals.
e) The standard compounds must be unreactive and stable in common liquid chromatography solvents.
f) The relationship between log *k* and the number of carbon atoms or characteristic functional groups in the molecules of the homologues must be linear.
g) They should not specifically interact with silica gel.
h) The *k* values should not depend on the mobile phase composition.

WIDELY USED RETENTION TIME INDEX SCALES IN LC

A great number of homologous series of compounds have been tested as retention index standards for HPLC, but most are only used by single laboratory. The most used retention index scales are 1-nitroalkanes, alkyl aryl ketones and alkan-2-ones [29], which are discussed later.

n-alkanes and n-alkylbencenes

As it has been commented before, n-alkanes were tested as retention index standards in HPLC, but they face the problem of being very nonpolar and therefore highly retained in RPLC. For this reason, most of the analytes of interest elute earlier than n-alkanes in HPLC systems. On the other hand, Kóvats retention index standards could not been detected by spectroscopic detectors and could only be detected by the use of refractive index detectors, which are temperature sensible and incompatible with gradient elution [27]. N-alkylbenzenes have the advantatge of being detectable spectroscopically, but they are relative non-polar and have the same problems than n-alkane standards [30].

Alkan-2-ones

Baker and Ma [31] developed the first retention index standards specifically for HPLC. They showed a linear correlation between the members of alkan-2-ones standards and their carbon number, even using a wide range of mobile phase composition. This retention index standards have a chromophore ketone group (C=O),

so they could be detected spectroscopically. Compared with n-alkylbenzenes and n-alkanes, retention of the most polar components of the retention index standards are compatible with R_t of polar drugs [1].

Alkyl aryl ketones

Alkyl aryl ketones were suggested as set of internal standards by Kikta and Stange [21]. Alkyl aryl ketones showed a linear relationship between log *k* and carbon number, as showed by Smith [28]. He proposed alkyl aryl ketones as more preferable standards than alkan-2-ones, as alkyl aryl ketones have stronger chromophores.

1-nitroalkanes

The most used retention index scale was proposed by Bogusz and Aderjan [32]. This retention index scale uses 1-nitroalkanes as retention index standards. They have a strong chromophore group (NO_2) and relatively high polarity in the smallest members of the homologous series. In addition, nitroalkanes are less reactive than alkan-2-ones and can be stored for longer time [1]. It is recommended to use a diode array detector as detector.

Other retention index scales are peptide index scale (which is based in peptide hydrophobicity and was used to peptide retention prediction) [33], polyaromatic hydrocarbons retention index scale [34] and alkylparaben retention index scale [35]. A retention scale was also built with drugs eluting at regular R_t using gradient elution [36].

COMPARISION BETWEEN RETENTION TIME INDEX SCALES

There are few papers that compare the different retention time index scales proposed. Thus, Smith [28] compared alkyl aryl ketones with alkan-2-ones, and realized that alkan-2-ones were more appropriated for less retained drugs than alkyl aryl ketones, which presented the advantage of having a strong chromophore. In addition, he noticed that retention values for alkan-2-ones increased with organic modifier composition. In other work [37], the same author compared alkyl aryl ketones, alkan-2-ones and 1-nitroalkanes and, in the three series, methylene increment was similar, as predicted by Martin (Eq. 2). A similar effect was reported by Bogusz and Aderjan [32]. Smith and Finn [37]calculate retention index of test compounds and different drugs using diverse mobile phases (methanol-water and acetonitrile-water), different pH values and

different stationary phases (octadecylsilica, C_{18} and octylsilica, C_8). One of the more striking results was that alkyl aryl ketones were more retained than aliphatic standards, so it was necessary to extrapolate retention indices in alkyl aryl ketones standards. On the other side, changes in retention due to changes in separation parameters such as pH or mobile phase composition can be partially mitigated by retention index, but it still necessary to control chromatographic conditions [32,38]. Didaoui [39] reached a similar conclusion when trying to test different methods to calculate retention index.

The different retention index standards behave differently when experimental conditions change, for this reason, secondary standards are introduced to correct retention indices [29,40,41].

PROBLEMS OF USING RETENTION TIME INDEX SCALES

Different problems need to be addressed before using retention time indices. The first problem is to select appropriate retention index standards, this is, compounds with retentions similar to analytes. In addition, it is difficult to find an universal retention index scale, suitable for all analyte, as retention index standard has to be similar to the analyte regarding functional groups to ensure that changes in chromatographic conditions affect in the same extension to the analyte and the retention index standards [1,42]. Other problems are possible differences in retention times when using diverse mobile phases or chromatographic conditions and the need of using secondary standards.

Retention index scales are typically used for identification of unknown compounds using spectrophotometric detectors [29,40,43,44], although they are also used to predict compound retention with confirmation purposes [45], to predict retention changes of some compounds due to the addition of groups like OH [46] or to characterize solute and solvents in RPLC [47]. The last applications are of some interest in the development of retention time prediction by using artificial neural networks (ANN).

2. Objectives

The aim of this work is to develop a retention time index scale to overcome absolute retention time variations in the chromatographic separation from

chromatographic conditions, analytical column aging as well as sample matrix in liquid chromatography coupled to high resolution mass spectrometry (LC-HRMS).

3. Experimental Section

REAGENTS AND STANDARDS

To synthesize 2-Amino-4-alkylphenol retention time index standards, 1-bromobutane, 1-bromopentane, 1-bromooctane, 1-bromododecane, triphenylphosphine, Palladium on carbon (Pd/C), potassium carbonate and 4-Hydroxy-3-nitrobenzaldehyde were purchased from Sigma-Aldrich (St. Louis, MO, USA). Toluene and 1,4-dioxane was supplied by Alfa Aesar (Karlsruhe, Germany).

Isotopically labeled standards and pesticide reference standards (Acetaminophen-d_4, Omeprazole-(5-methoxy-d_3), Diuron-d_6 Thiabendazole-d_6, Tebuconazole-d_6, Chlorpyrifos-d_{10}, Carbamazepine-10,11-epoxide-d_{10}, Irbesartan-d_6, Testosterone-$^{13}C_2$, Quizalofop-p-ethyl-d_3, Terbuthylazine, Simazine, Terbutryn, Terbumeton, Thiabendazole, Diuron, Carbendazim, Atrazine, Imazalil, Deetylterbumeton and Prochloraz) were brought from Sigma-Aldrich, Dr. Ehrenstorfer, and Fluka.

INSTRUMENTATION

A Waters Acquity UPLC system (Waters, Milford, MA, USA) was interfaced to a hybrid quadrupole-orthogonal acceleration-TOF mass spectrometer (XEVO G2 QTOF, Waters Micromass, Manchester, UK) (*Figure 6*), using a Z-spray ESI interface operating in positive ion mode. The chromatographic separation was performed using an Acquity UPLC BEH C18 1.7 µm particle size column 100 × 2.1 mm (Waters) at a flow rate of 300 µL/min. The mobile phases used were A = H_2O with 0.01% HCOOH and B = MeOH with 0.01% HCOOH. The initial percentage of B was 10%, which was linearly increased to 90% in 14 min, followed by a 2 min isocratic period and, then, returned to initial conditions during 2 min. The total run time was 18 minutes. Nitrogen was used as drying gas and nebulizing gas. The gas flow was set at 1000 L/h.

Figure 6. XEVO G2 QTOF.

TOF-MS resolution was approximately 20.000 at full width half maximum at *m/z* 556. MS data were acquired over an *m/z* range of 50–1000. A capillary voltage of 0.7 kV and cone voltage of 20 V were used. Collision gas was argon 99.995% (Praxair, Valencia, Spain). The desolvation temperature was set to 600 °C, and the source temperature to 120 °C. The column temperature was set to 40 °C.

For MS^E experiments, two acquisition functions with different collision energies were created. The low energy function, selecting a collision energy of 4 eV, and the high energy function, with a collision energy ramp ranging from 15 to 40 eV in order to obtain a greater range of fragment ions. The LE and HE functions settings were for both a scan time of 0.3 s.

2-AMINO-4-ALKYLPHENOL HOMOLOGOUS SERIES

One of the objectives of this work was to develop a retention time index scale based on a series of standards suitable for mass spectrometry detection in liquid chromatography.

The new homologous series needed to be ionizable by ESI source and capable to be detected in both positive and negative ionization mode (PI and NI, respectively). So it would be desired the presence of basic and acidic groups in the molecules. For this reason, an amino and a hydroxyl group were selected to be present in the basic skeleton of the standard series. To increase the acidity of the OH group, it was bounded to a benzene ring, forming a phenol group. Changes in R_t of each standard would therefore be given by the alkyl chain length. The general structure of aminoalkylphenol series is given in *Figure* 7.

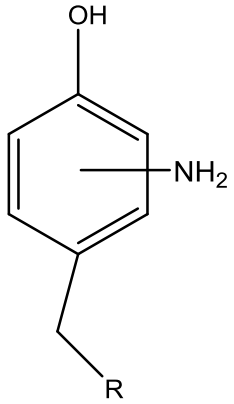

Figure 7. Aminoalkylphenol general structure.

Arbitrarily, 2-amino-4-alkylphenols were selected as standards which were then searched in Pubchem [48] to obtain the hydrophobicity of the series to ensure the covering of all the chromatographic range. Information about 2-amino-4-alkylphenols selected is showed in *Table 1*.

Table 1. 2-amino-4-alkylphenols information.

Compound	Molecular Formula	MW (g/mol)	Log P
2-aminophenol	C_6H_7NO	109.1259	0.6
2-amino-4-methylphenol	C_7H_9NO	123.1525	1.2
2-amino-4-ethylphenol	$C_8H_{11}NO$	137.1790	1.6
2-amino-4-propylphenol	$C_9H_{13}NO$	151.2056	2.1
2-amino-4-butylphenol	$C_{10}H_{15}NO$	165.2322	2.6
2-amino-4-pentylphenol	$C_{11}H_{17}NO$	179.2588	3.2
2-amino-4-hexylphenol	$C_{12}H_{19}NO$	193.2854	3.8
2-amino-4-heptylphenol	$C_{13}H_{21}NO$	207.3119	4.4
2-amino-4-octylphenol	$C_{14}H_{23}NO$	221.3385	4.8
2-amino-4-nonylphenol	$C_{15}H_{25}NO$	235.3651	5.4
2-amino-4-decylphenol	$C_{16}H_{27}NO$	249.3917	6
2-amino-4-undecylphenol	$C_{17}H_{29}NO$	263.4183	6.6
2-amino-4-dodecylphenol	$C_{18}H_{31}NO$	277.4448	7.2

Due to the high price of some of the standards unavailability of some standards of the retention time index scale, it was decided to in-house synthesize some of these compounds: 2-amino-4-ethylphenol, 2-amino-4-pentylphenol, 2-amino-4-hexylphenol, 2-amino-4-nonylphenol and 2-amino-4-tridecylphenol.

In order to synthesize the homologous series of 2-amino-4-alkylphenol, a retrosynthetic analysis was carried out (*Figure 8*). After an extensive study of the three

potential routes, the third way was selected (route c). To obtain the desired product, the route c was defined as a three step process (*Figure 9*): (1) phosphonium salt can be obtained from an alkyl halide and triphenylphosphine. (2) Wittig reaction can be used in the second step to form the alkene from the aldehyde using the phosphonium salt. (3) Finally, the third step involves the reduction of the alkene to obtain the final alkane.

The Wittig reaction is a widely studied reaction used to obtain an alkene from an aldehyde or ketone since its publishing in 1954 [49] and it is very used worldwide due to its high reaction yield.

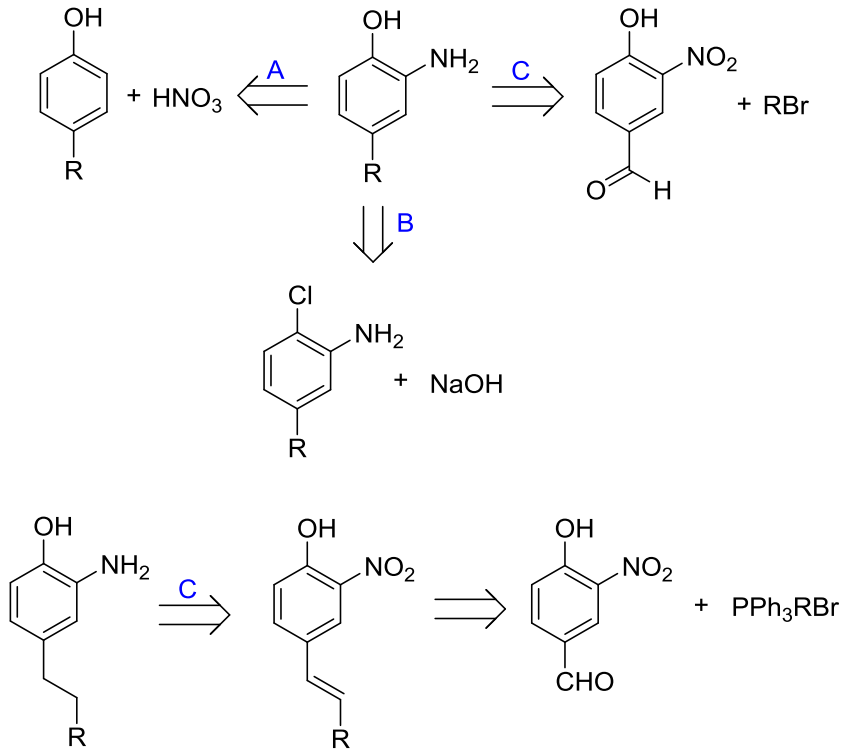

Figure 8. Retrosynthesis of possible routes and selected to obtain 2-amino-4-alkylphenol.

The first stage was to develop the general procedure to synthesize the phosphonium salts. In this way, triphenylphosphine (1.34 g, 4.6 mmol) and 1-bromobutane (0.5 mL, 4.6 mmol) were placed in a falkon tube and dissolved in 5 mL of acetone (*Table 2, entry 1*). After 12 h, the expected precipitate was not formed, so the mixture was placed in an ice bath to force the precipitation. However, no precipitate appeared. Then, the same procedure was repeated, but using methanol instead of acetone (*Table 2, entry 2*). No differences were observed. Thus, after a bibliographic search, the conditions recommended by Guan [50] was applied using toluene as solvent: a mixture of triphenylphosphine (1.34 g, 4.6 mmol) and 1-bromobutane (0.5 mL, 4.6

mmol) dissolved in toluene (3 mL) was heated under reflux for 8 h (*Table 2, entry 3*). Then, the mixture was cooled to room temperature and washed with diethyl ether to precipitate the desired phosphonium salt. After that, the solid was dried to perform a ^{1}H-NMR, but the data showed that the reaction was not completed and the reaction time was then increased. The final procedure (*Table 2, entry 4*) is described later.

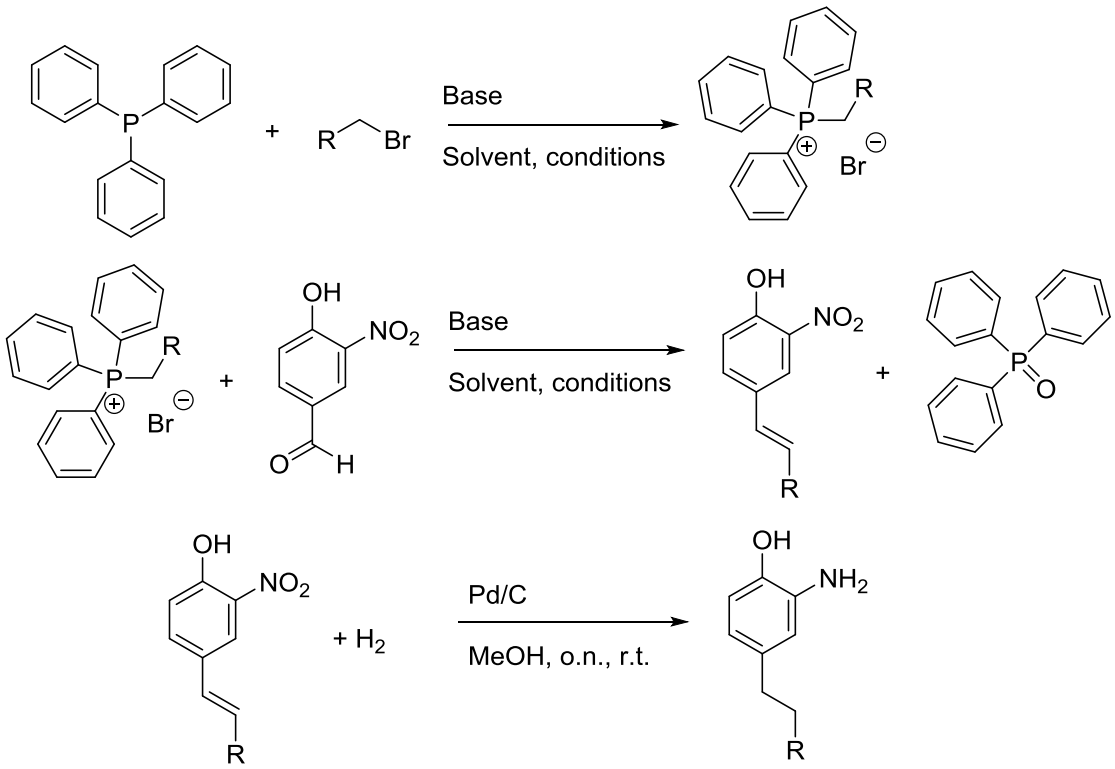

Figure 9. Selected procedure.

Table 2. Conditions and observations for each reaction.

Entry	Solvent	Conditions	Observation
1	Acetone	12 h / r.t.	No product
2	MeOH	12 h / r.t.	No product
3	Toluene	8 h / reflux	Product
4	Toluene	48 h / reflux	Product

General procedure to form phosphonium salts

The procedure to prepare the phosphonium salts (*Figure 10*) were developed based on the works done by Guan [50] and Palecek [51]. The procedure is as follows: in a round bottom flask, the appropriate amount of triphenylphosphine and bromoalkane were weighed. Then, they were dissolved in toluene, stirred vigorously and refluxed for 48 h. After that, solvent was evaporated in a rotovapor and the resulting solid washed

with Et_2O until a white solid appears. Finally, the solid was filtered. Identification and characterization of the phosphonium salts was carried out by ^1H NMR and MS.

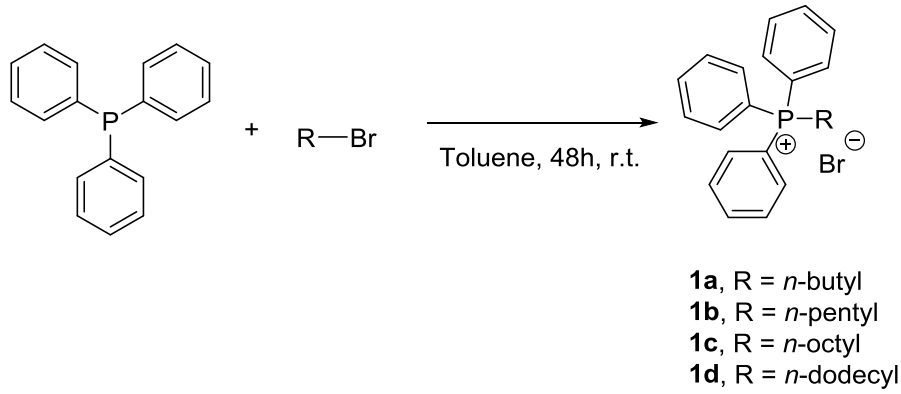

Figure 10. General procedure to form phosphonium salts.

Synthesis of n-Butyl(triphenyl)phosphonium bromide (1a)

1-Bromobutane (3 mL, 28 mmol), triphenylphosphine (7.531g, 28 mmol) and toluene (42 mL) were placed in a round bottom flask to synthetize n-butyl(triphenyl)phosphonium bromide, following the general procedure to form phosphonium salts. Mass spectra for **1a** are showed in *Figure 11*.

Synthesis of n-pentyl(triphenyl)phosphonium bromide (1b)

1-Bromopentane (3 mL, 24 mmol), triphenylphosphine (6.378 g, 24 mmol) and toluene (36 mL) were placed in a round bottom flask to synthetize n-pentyl(triphenyl)phosphonium bromide, following the general procedure to form phosphonium salts.

Synthesis of n-octyl(triphenyl)phosphonium bromide (1c)

1-Bromooctane (3 mL, 17 mmol), triphenylphosphine (4.561 g, 17 mmol) and toluene (27 mL) were placed in a round bottom flask to synthetize n-octyl(triphenyl)phosphonium bromide, following the general procedure to form phosphonium salts. N-octyl(triphenyl)phosphonium bromide was isolated as an oil.

Synthesis of n-dodecyl(triphenyl)phosphonium bromide (1d)

1-Bromododecane (3 mL, 12 mmol), triphenylphosphine (3.295 g, 12 mmol) and toluene (18 mL) were placed in a round bottom flask to synthetize n-

dodecyl(triphenyl)phosphonium bromide, following the general procedure to form phosphonium salts.

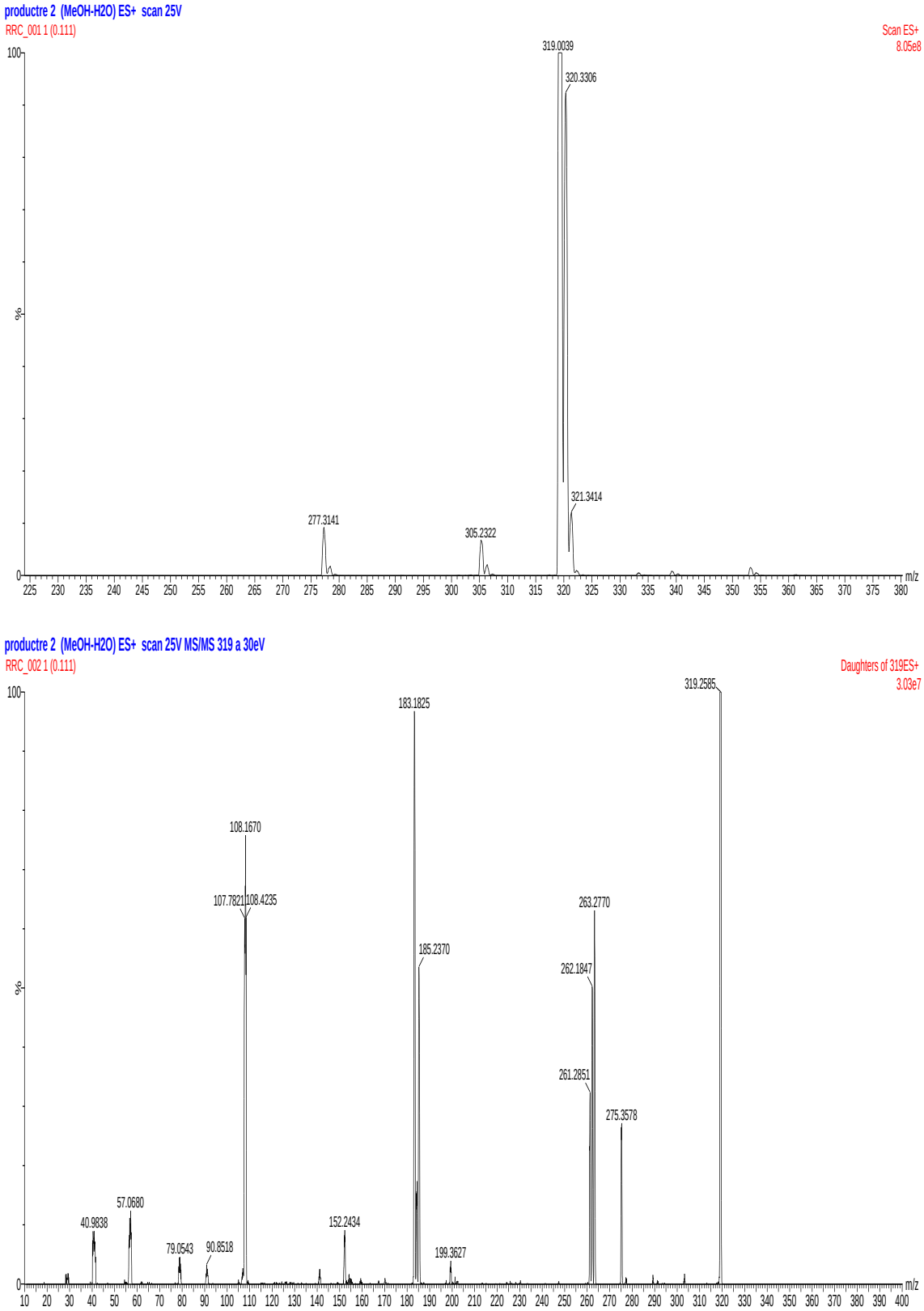

Figure 11. Full scan of product from reaction 1a (up). Doughter scan corresponding to n-Butyl(triphenyl)phosphonium bromide (m/z=319) (below).

Wittig reaction general procedure

The general procedure to the Wittig reaction was taken from Le Bigot [52]. In a round bottom flask a phosphonium salt **1a** (0.1 g, 0.25 mmol) was dissolved in MeOH (0.2 mL). Then Na_2CO_3 (61 mg, 0.6 mmol) was added and stirred vigorously for 10 min. After that time, benzaldehyde (0.02 mL, 0.2 mmol) (it was used benzaldehyde to check the best condition for Wittig reaction) was added and stirred for 2 h at room temperature (*Table 3, entry 1*). On the other hand, the same reaction was done using NaOH (0.133 g, 0.325 mmol) as base (*Table 3, entry 2*). The same reaction using MeOH and NaOH was also performed but heated at 30ºC (*Table 3, entry 3*). Finally, another reaction conditions were evaluated using MeOH but double NaOH (0.26 g, 0.65 mmol) was employed (*Table 3, entry 4*). All the reactions were carried out using the same amount of methanol, phosphonium salt and benzaldehyde, and the same reaction time. The four reactions were stopped with NH_4Cl and products isolated by a liquid-liquid extraction using dichloromethane (30 mL). The reactions yield were checked by ^{1}H-NMR. The last reaction was the most successful (*Table 3, entry 4*). These conditions were selected but using now the aldehyde of interest, 4-hydroxy-3-nitrobenzaldehyde (0.1725g, 1 mmol) for Wittig reaction, but unfortunately no product was detected.

The last prove was done following the procedure described by Le Bigot using 1,4-dioxane [52]. In a round bottom flask, phosphonium salt **1a** (0.398 g, 1.25 mmol) were dissolved in 1,4-dioxane (1 mL) and stirred vigorously for 30 min. Then, K_2CO_3 (0.208 g, 1.5 mmol) was added to the mixture and after 15 min, 4-hydroxy-3-nitrobenzaldehyde (0.172 g, 1 mmol) was added and heated to 90ºC for 30 min. After that, the reaction was stopped and the solvent evaporated in a rotovapor. The solid was characterized by ^{1}H-NMR, and the expected product was detected but as a minor compound.

Table 3. Conditions of Wittig reaction proves.

Entry	Base / Solvent	Conditions
1	Na_2CO_3, MeOH	2 h / r.t.
2	NaOH, MeOH	2 h / r.t.
3	NaOH, MeOH	2 h / 30ºC
4	NaOH (2eq), MeOH	2 h / 30ºC

Due to the lack of time to optimize Wittig reaction conditions and the optimization of the subsequent hydrogenation step, the in-house synthesis for developing a retention index based on 2-amino-4-alkylphenol homologues has unfortunately to be postponed.

Presumably, next summer, synthesis will be ended and then chromatographic and mass spectrometric behavior of 2-amino-4-alkylphenols will be tested. In addition, 2-amino-4-alkylphenol retention time index will be calculated and tested in different matrices and in different columns, preferably with new and aged columns to test if retention indices can overcome changes in retention.

LABELED COMPOUNDS RETENTION INDEX SCALE

Another option to develop a retention index scale for LC-MS is to use a set of different compounds available on the laboratory which elute at constant different R_t, like retention time scale developed by Elliot [36]. In this way, a group of isotopically labeled compounds was selected to ensure that retention time index standards are not present in the samples. Within this set of standards were present drugs, pharmaceuticals and pesticides, all ionized in positive mode (see *Table 4*).

Table 4. Selected isotopically labeled standards and retention time of each standard.

	R_t
Acetaminophen-d_4	2.3
Thiabendazol-d_6	4.11
Omeprazole-d_3	7.55
Carbamazepine 10,11 epoxide -d_{10}	8.65
Diuron-d_6	9.56
Irbesartan-d_6	10.62
Testosterone-$^{13}C_2$	11.63
Tebuconazole-d_6	12.33
Quizalofop-p-ethyl-d_3	13.46
Chlorpyriphos-d_{10}	14.06

A mixture was made using selected isotopically labeled standards with a concentration of 0.1 ppm and stored until injection into the UHPLC-(ESI)QTOF MS system.

APPLICATION TO DIFFERENT MATRICES.

In order to check the suitability of this approach, different matrices were tested. Thus, five water samples taken from Castelló area (effluent waste water, influent waste water, sewage water (leachate from urban solid waste), ground water and river water) as well as, a urine and a tomato extract were used. Water samples were simply centrifuged whereas tomato extract was filtered and fivefold diluted and urine was centrifuged and diluted twice before being fortified.

Fortified samples were spiked using both a pesticide mix (0.5 ppm) (see *Table* 5) and the mixture made with the selected isotopically labeled standards (0.5 ppm) to achieve a final concentration of 0.1 ppm.

At the time of writing, the isotopically labeled retention index scale standards and samples could not be injected into the UHPLC-(ESI)QTOF MS system, as regrettably there was a problem with the UHPLC pump. Next steps to follow in the development of this retention index scale would consist in the calculation of the retention index of the spiked pesticides to ensure that pesticides and isotopically labeled standards are affected in the same extent due to changes in matrix composition and analytical column aging, showing that retention index are truly more consistent than retention time over time.

Table 5. Pesticides present in Mix.

Pesticide
Terbuthylazine
Simazine
Terbutryn
Terbumeton
Thiabendazol
Diuron
Carbendazim
Atrazine
Imazalil
Deethylterbumeton
Prochloraz

If the last experiments were successful, another retention time index scale based on isotopically labeled compounds, but in negative ionization mode, will be prepared and evaluated.

4. Conclusions

A linear correlation between retention time and alkyl chain carbon number is expected for 2-amino-4-alkylphenol. This is a retention index scale built with easily synthesizable standards. Although these standards contradict Smith criteria a and b, they will be detected without problem using MS and probably with UV due to the aromatic ring.

On the other hand, isotopically labeled retention index scale presents some drawbacks such as needing previous information about the retention time of the standards in order to select the appropriate ones and their high cost. However, advantageously, they will not be present at any sample.

We hope that retention time index scale based on 2-amino-4-alkylphenol standards and isotopically labeled standards will reduce retention time variations produced by changes in matrix composition and column aging. It would be a very useful tool for avoiding false negative reports.

5. Acknowledgements

The author wants to thank the help received by Florenci González and Antonio Latorre from the Organic chemistry department on the synthesis development process.

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