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Doctor of Philosophy in Chemical Sciences

USE OF ADVANCED ANALYTICAL METHODS FOR THE INVESTIGATION OF REAL WORLD SAMPLES, WITH FOCUS ON ECO-SUSTAINABLE APPROACHES

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Scope of the research work

My research work, during the Ph.D. course, has been focused mainly on the development of advanced and innovative chromatographic techniques as an alternative to traditional liquid chromatographic techniques for complex sample analysis.

In particular, the present thesis focused on the development of sustainable analytical approaches that reduce or eliminate the use of organic solvents in order to minimize the production of waste, and ensure the safety of the operator and the environment.

Also, the level of toxic contaminants and essential molecules in the waste products of fishery industry was evaluated for the production of dietary supplements for human consumption, enhancing the concept of recycling, in accordance with a circular economy theory or, specifically, with the concept of Blue Economy.

The techniques employed in this thesis were: hand-portable capillary liquid chromatography (Cap-LC), high temperature liquid chromatography (HTLC) or superheated water chromatography (SHWC), and inductively coupled plasma-mass spectrometry (ICP-MS).

The developed methods have been applied to food, cosmetic and forensic samples (tuna, candy, blush and inflorescence of cannabis).

Advanced technologies, including a prototype system, namely the HTLC instrumental setup, combined with the innovative analytical approaches enabled the obtainment of satisfactory results for the identification of target analytes, often fulfilling the principles of Green Analytical Chemistry: on-site analyses, miniaturization, use of green solvents, multianalyte methods and high sample throughput.

Chapter 1

High Performance Liquid Chromatography

1.1. Introduction

Chromatography is a physical method of separation, in which the components of the sample are selectively distributed between two immiscible phases.

The "official" definitions of the International Union of Pure and Applied Chemistry (IUPAC) are: "Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (stationary phase) while the other (the mobile phase) moves in a definite direction. Elution chromatography is a procedure in which the mobile phase is continuously passed through or along the chromatographic bed and the sample is fed into the system as a finite slug" [1].

The history of chromatography dates back to the early twentieth century, when the Russian botanist Michail Tswett (1872–1919) used a column packed with a calcium carbonate stationary phase to separate colored pigments from plant extracts [2].

Michail Tswett observed that the different colored pigments, which initially formed the chlorophyll, were transported by mobile phase (petroleum ether) and retained with different affinities on the stationary phase ($CaCO_3$), this determined the formation of various differently colored bands.

Tswett is credited as being the "father of chromatography", he first used the term chromatography, combined the Greek word "χρώμα" color and "γραφή" graphy.

In the beginning, chromatography was not accepted by the academic community; only in 1941, thanks to research conducted by Martin and Synge, this innovative and brilliant idea is accepted and they were awarded the 1952 Nobel Prize in chemistry for this work [3].

Chromatographic separation may be classified by the physical state of stationary phase and mobile phase used, by the type of contact between the mobile phase and the stationary phase; or by the chemical or physical mechanism responsible for separating the analytes.

Chromatographic techniques are classified into three types based on the physical state of mobile phase used in: Gas Chromatography (GC), Liquid Chromatography (LC) and Supercritical Fluid Chromatography (SFC).

The classification of main chromatographic techniques is shown in Figure 1.1.

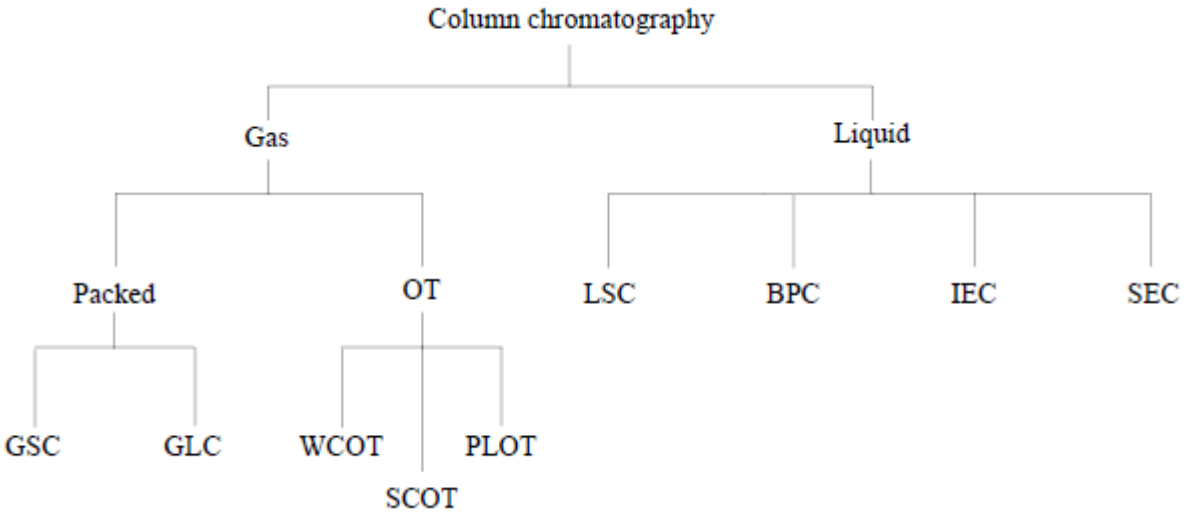

Figure 1.1. *Summary scheme of chromatographic techniques. Abbreviations: OT - open tubular; WCOT - wall coated open tubular; SCOT - support coated open tubular; PLOT - porous layer open tubular; LSC - liquid-solid chromatography; BPL - bonded-phase chromatography; IEC - ion exchange chromatography; SEC - size exclusion chromatography.*

In recent years, High Pressure Liquid Chromatography (HPLC) has been driven by the development of a number of research area such as food omics, biology and environmental analysis. This and following chapters are devoted to recent developments and approaches in liquid chromatography.

HPLC is a physical separation technique in which a liquid is the mobile phase. HPLC is a major analytic tool in many scientific areas and has several applications in fields of food analysis, scientific investigation, environmental analysis.

The technique is applicable to diverse compounds, from small organic compounds and ions to large biomolecules, without any limitation of volatility or thermal stability.

The principles of HPLC is the same as that of liquid chromatography (LC), but this is the most recent development. This “evolution” is characterized by:

- small diameter (2-5 mm);
- the stainless-steel columns can be used repeatedly;
- column packings with very small (3, 5 and 10 μm) particles and the continual development of new substances to be used as stationary phases;
- relatively high inlet pressures and controlled flow rate of the mobile phase;
- small sample size and precise sample introduction;
- special continuous flow detectors capable of handling small flow rates and detecting very small amounts;
- automated standardized instruments;

- fast analysis speed;
- high resolution.

The indispensable parts of HPLC are represented in Figure 1.2.

The HPLC instrumentation is equipped with one or more solvent reservoir, connected with pumps that produce a constant flow rate at high pressure. The composition of mobile phase might be kept constant (isocratic mode) or changed (gradient mode) during the chromatographic separation.

The sample is introduced using an autosampler or syringe into the sample injector. Small quantities of sample, in a highly reproducible way, are transferred from the injector to the chromatographic column, where separation occurs through the continuous redistribution between the mobile and stationary phases. A detector, situated at the column outlet, should be able of monitoring the analytes as they elute from the chromatographic column. A data collection device allows to store and reprocess chromatographic information.

The column is the most important part of instrumentation, because inside of it the physiochemical process of separation occurs. The quality of separations depends primarily on the nature of stationary phase, particle diameter and packed material utilized. Generally, the columns are placed inside the oven to keep the temperature constant during the analysis.

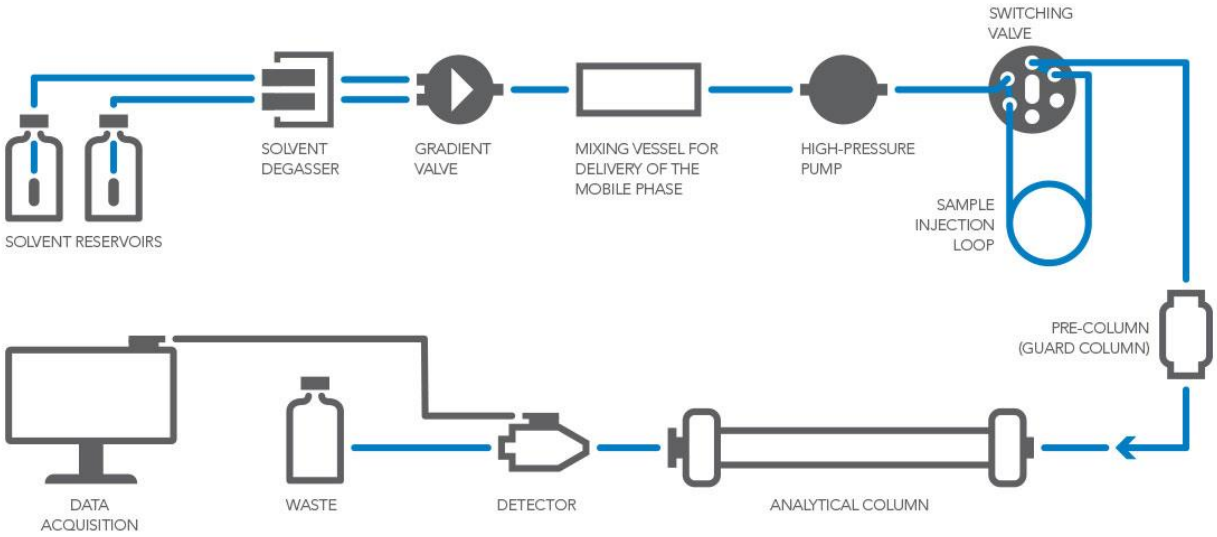

Figure 1.2. *The main components of high performance of liquid chromatography.*

High Performance Liquid Chromatography offers a wide variety of separation mechanisms, such as normal phase (NP), reversed phase (RP), size exclusion (SEC) and ion exchange (IEC) characterized by different selectivities:

- Normal phase: Is the oldest liquid chromatography separation modes. The phase system consists of a polar stationary phase and a non-polar or moderately-polar mobile phase. The

retention of the sample components to be separated is determined by the interaction of the polar surface of the stationary phase with the dissolved analyte in the mobile phase.

- Reversed phase: The phase system consists of a non-polar stationary phase and a polar mobile phase (usually aqueous-organic solvents). A non-polar chemically modified (frequently with octadecyl (-$C_{18}H_{37}$) groups) silica gel is the most widespread stationary phase.
- Size-exclusion or gel-permeation chromatography (SEC): porous gels are used as stationary phases, and the molecules in solution are separated based on the difference in the size. Large solutes pass quickly because not enter into the porous stationary phase, smaller solutes enter into the porous stationary phase, increasing the time spent on the column.
- Ion-exchange chromatography (IEC): The ion exchange process represents an equilibrium reaction; the ionic solutes are attached to the stationary phase by electrostatic forces. The stationary phases consisting of a solid support with covalently attached anionic (e.g., $–SO_3^–$) or cationic (e.g., $–N(CH_3)^{3+}$) functional groups are used. When an organic salt (ion pair reagent) is added to the mobile phase we obtain ion pair chromatography which is an alternative to ion exchange chromatography. An organic ionic substance is added to the mobile phase and forms an ion pair with a sample component of opposite charge.

1.2. Mass Spectrometry and the benefits of using the Linear Retention Indices (LRI) in LC analysis†

1.2.1. Introduction

Mass spectrometry (MS) is the most important analytical tool for the characterization of organic molecules.

Mass spectrometry (MS) is a technique based on the ionization of the sample and the ions are separated according to their mass-to-charge ratio (m/z) and detected in proportion to their abundance, in a high vacuum system. It provides this result as a plot of ion abundance versus mass-to-charge ratio. At present, MS is the most sensitive method for molecular analysis and it is the most used technique coupled to the chromatographic systems, gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS), due to the high sensitivity and specificity, high throughput and high accuracy. To date, the most common ionization techniques in HPLC are atmospheric pressure, such as electrospray ionization (known as ESI or APESI) and chemical ionization at atmospheric pressure (known as APCI). In ionization at atmospheric pressure the molecules are at first ionized at atmospheric pressure and then separated mechanically or electrostatically from neutral molecules.

The most use mass analyzers, mainly because a low-moderate economic cost, are quadrupole (Q), ion-trap (IT) and time-of-flight (ToF), beside a pool of hybrid instruments auch as Q-ToF, IT-ToF and QqQ (triple quadrupole). In recent decades, the mass spectrometer (MS) has become the detector of choice for obtaining structural information on unknown peaks, thanks to the elucidation of fragments, often originating from the loss of specific functional groups; however, the cost and the level of experience of the operators is certainly higher than the use of retention data. In the following section we will discuss the advantages of using the Linear Retention Index (LRI) in LC, the requirements and the main challenges, also compared to gas chromatography methods, where the LRI is stably used for identification purposes, usually in combination with spectral libraries MS.

Currently, mass spectrometry (MS) plays a key role in several application fields due to its characterization and identification potential. In fact, it offers the possibility to determinate unknown structures and to elucidate complex mixtures, particularly if hyphenated with chromatographic techniques. Certainly, electron ionization (EI) sources perfectly fit such a purpose because of a typical fragmentation pattern generated by the impact between gas-phase particles and an accelerated electron beam at 70 eV energy under high vacuum conditions. Under these conditions, matrix effects are completely avoided and high spectral reproducibility is ensured, so that EI-MS spectra acquired in the present will perfectly match with spectra acquired in the past and in another laboratory. This allows for the extensive use of EI- MS spectral libraries, largely available on the market and in costant

implementation, thus achieving a fast, automatic and highly reliable identification of unknown samples. Nevertheless, EI-MS applicability is limited to small compounds, typically up to 600 Da, characterized by a certain degree of volatility and a high/moderate thermal stability. It represents the detector of choice for gas chromatography (GC) systems, which are quite compatible with the EI operation mode. Contrarily, higher molecular weight compounds such as many biomolecules, for example lipids, polyphenols, carotenoids, and other pigments, are typically analyzed by liquid chromatography (LC) methods, whose solvent amounts are much more in agreement with atmospheric pressure ionization (API) MS interfaces, mainly based on chemical or electro-induced ionization. Solvents and eventually organic modifiers utilized in the mobile phase play a critical role in the formation of ions as charge carriers or conductivity enhancers, but the high mobile phase-analyte interaction is responsible for a remarkable matrix effect that represents the Achille's heel of LC-MS analytical approaches. Furthermore, mobile phase or sample impurity can influence the ion formation process, consequently altering the resulting MS spectrum. For this reason, no API-MS spectral database is commercially available or online and the matter of identification is currently a big challenge for LC users. In addition, although API-MS techniques take advantage of the preservation of the molecule-related ion, viz. the molecular weight information, almost no fragments are produced because of the low energy involved in the process. Consequently, the acquired spectra are poorly informative and more advanced MS instruments, able to perform MS/MS experiments, are necessary for structure elucidation. Even MS/MS libraries cannot be used at inter-laboratory levels since they are strongly dependent by the optimized collision energies and voltages, which can be different by changing the instrumental configuration or instrument brand. Two different solutions were recently suggested to overcome the issue of identification in LC:

1) The miniaturization of the LC part to be coupled with EI-MS has resulted in the development of a prototype called Nano LC-EI-MS [4–6], whose main limitation is related to the difficult vaporization and desolvation process of higher boiling compounds at the LC-EI-MS interface, which can lead to a memory effect. The EI source was then extended to semi-volatile compounds, for example, polymethoxyflavones and coumarins [7,8] or free fatty acids [9–11], despite the difficulty of vaporization and ionization of larger molecules.

2) To achieve reliable retention data at inter-laboratory levels, the Linear Retention Index (LRI) approach was used. This method is commonly used in GC to obtain unequivocal identification of unknowns. The LRI values can be included into the EI-MS spectral libraries to provide a dual-filter spectral search, allowing to distinguish of compounds with similar MS spectrum using retention data. Despite numerous attempts in the 1980s and 1990s to use the LRI identification system in LC [12–17], the lower repeatability of retention times compared to GC, the broader range of LC-amenable

compounds, the larger number of elution options (different mobile phase composition), and differences in instrumentation and column packaging limited the use of LRI databases at both the intra- and inter-laboratory levels [19–22]. Since the LRI is a relative or normalized retention time with respect to standard compounds eluting in the same elution window, the reference compounds used play a crucial role. Unlike the relative retention time method, which uses a single standard compound for normalization purposes, the LRI approach compares the retention time of an analyte to that of two standard compounds eluted immediately before and after it, thus ensuring a more accurate compensation of instrumental and/or analytical variations. For the determination of LRIs in complex mixtures, which are often analyzed using chromatographic techniques, a mixture of reference compounds covering the entire chromatographic space is required. In GC, since the elution occurs basically according to increasing boiling point, it is not difficult to obtain a suitable reference homologue series; mixtures of alkanes or fatty acid methyl esters have been used effectively [23–27]. In LC, where the three interactions analyte-mobile phase-stationary phase play a major role in the elution process, each application could need a dedicated reference series, satisfactorily eluted under the employed conditions, e.g. aqueous or non-aqueous reversed phase (NARP) LC. The difference between the identification processes in GC and LC platforms, which represents the starting point of this study, is summarized in Fig. 1.3, while the following sections will be dedicated to an in-depth explanation of the LRI concept, the requirements for the proper functioning of an identification process based on it, and different developed applications in the LC field, including some papers that combine LRI and spectral database. One of them, through the use of the nano LC-EI-MS prototype, combined the LRI values into an EI-MS library, utilizing fast, automatic and reliable identification process of GC-MS (Fig.1.3.) for the first time in LC.

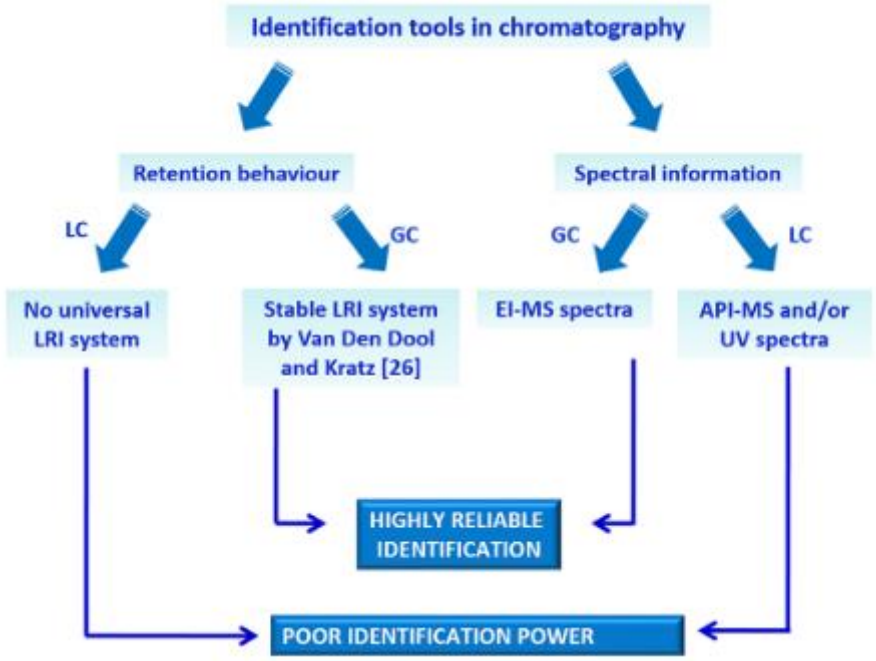

Figure 1.3. *Scheme of the identification process in chromatography.*

1.2.2. The Linear Retention Index concept

Kovàts [28] introduced the retention index (RI) system and suggested the use of a homologous series of n-alkanes to fix the GC retention behaviour of analytes, which are analysed under the same experimental conditions. Each standard compound's RI value is assigned according to the number of carbon atoms in the alkyl chain. To avoid using a decimal fraction, the number of carbon atoms is conventionally multiplied by 100 (e.g., the retention index is 1200 for dodecane). The RI of a given compound is equal to the number of carbon atoms (x 100) of a hypothetical standard compound having the same adjusted retention time as that given compound. For example, a compound with a RI equal to 1290 elutes between the alkanes dodecane (I = 1200) and tridecane (I = 1300). When isothermal GC conditions are applied, the homologous series elutes with retention times increasing exponentially along with the alkane chain length. As a results, the retention time and the RI of the reference alkanes have a logarithmic relationship, as shown by the equation:

$$I = 100\left[z + \frac{logt'_{Ri} - logt'_{Rz}}{logt'_{R(z+1)} - logt'_{Rz}}\right] \qquad [Eq.\,1.1]$$

t'_{Ri} is the adjusted retention time of the unknown compound, t'_{Rz} e $t'_{R(z+1)}$ are the adjusted retention times of the alkanes which elute, respectively, immediately before and after the unknown compound, and z represents the number of carbon atoms in the alkane chain (i.e., chain length) of the alkane eluted immediately before the unknown peak. Instead of using the retention time (t_R), the use of the adjusted retention time ($t'_R = t_R - t_M$), where t_R is the retention time of the analyte and t_M is the

retention time of an unretained compound, is used to reduce the influence of the mobile phase-analyte interaction and its effect on the extracolumn volume. In GC this is achievable since the carrier gas has no effect on relative retention. This assumption cannot be extended in LC, which makes the application of the RI approach in LC methods problematic. In fact, the liquid mobile phase interacts strongly with the stationary phase, forming a solvation sphere around its functional groups and decreasing the accessibility of the analyte inside the column. This means that changing the mobile phase flow rate can have a distinct effect on highly retained compounds *vs* poorly retained ones. Because the retention times of n-alkanes increase linearly with the number of carbon in temperature programmed GC, the logarithmic expressions in Eq. (1.1) were removed, and a linear relationship between retention times (t_R) and RIs (hereinafter LRIs) was established, according to the equation proposed by van den Dool and Kratz [29] :

$$LRI = 100\left[z + \frac{t_{Ri} - t_{Rz}}{t_{R(z+1)} - t_{Rz}}\right] \qquad [Eq.\,1.2]$$

wherein the adjusted retention times were replaced by the uncorrected retention times due to mathematical simplification of the t_M term. This approach has also been proposed using polycyclic aromatic hydrocarbons as homologous series [30]. In this article, Lee et al. reported that a RI system based on closely related compounds as internal standards was more reliable for polycyclic aromatic hydrocarbons analysis by GC. In other words, if the reference compounds have chemical-physical characteristics comparable to those of the analytes to be measured, the reproducibility of the retention indices improves when they are similar to those of the analytes to be determined. Other LRI systems have been described in the literature as a result of this consideration. The one that uses fatty acid methyl esters with an even carbon chain number as the homologous series for fatty acids identification by GC [31,32] was the most widely used. In this case, as the difference of carbon atoms between two subsequent compounds of the homologous series is equal to two, Eq. (1.2) is modified as follows:

$$LRI = 100\left[z + 2\frac{t_{Ri} - t_{Rz}}{t_{R(z+2)} - t_{Rz}}\right] \qquad [Eq.\,1.3]$$

Therefore, a generalization for LRI calculation can be performed according to Eq. (1.4)

$$LRI = 100\left[z + n\frac{t_{Ri} - t_{Rz}}{t_{R(z+n)} - t_{Rz}}\right] \qquad [Eq.\,1.4]$$

where n represents the difference in z units between the reference compounds eluted immediately before and after the unknown analyte.

1.2.3. Requirements of the ideal homologue series in LC

The ideal homologue series for LRI calculation should meet the following criteria:

1) Full coverage of the chromatographic space is one of the most difficult problems in LC due to the large number of LC-amenable compounds compared to the small number of natural and synthetic chemicals tipically analysed by GC. Within this context, the common practice of derivatization processes, which transform polar functional groups into less polar chemical functions prior to GC injection, reduces the chemical interaction with the stationary phase, allowing compounds to be eluted based on their vapour pressures. Thus, the only requirement for a GC reference homologue series is the proper coverage of the boiling temperatures of the unknowns. On the other hand, in LC, the usual practice of analysing the compounds, e.g. biomolecules, in their intact form considerably increases the sphere of chemical properties, such as polarity, water solubility, acidity, etc., which results in the increase of the chromatographic space, viz. elution range. As a result, gradient elution is required to provide a variation of the mobile phase composition, able to elute both polar and non-polar compounds, acidic or non-acidic molecules, etc. Therefore, the ideal reference homologue series should include substances that can be eluted under a wide range of elution conditions, e.g., from 100% of water to 100% of organic solvent. It's worth noting that finding homologues that properly cover the entire chromatogram is only possible under reversed phase (RP) conditions, because the addition of one or more methylene groups causes a uniform change in hydrophobicity, which is directly correlated to the retention on a RP column, whereas other separation modes are based on specific interactions and are less influenced by hydrophobicity.

2) Similar retention properties of the target analytes, so that the retention behaviour of the reference compounds change in a similar way to that of the analytes on changing the operating conditions, such as the flow rate and/or the gradient steepness. This means that reference compounds and analytes should have similar chemical and physical properties in order to have comparable chemical interactions with both stationary and mobile phase. In this way, the LRI will remain constant even if the retention time changes. Such a requirement represents the biggest challenge in LC because of the wider range of retention properties depending on many chemical/physical interactions, so that some compounds can have a stronger interaction with the stationary phase and others with the mobile phase: the first will be less affected by variations of the flow rates and gradient steepness with respect to the latter.

3) Intense signals on the detectors used are chosen according to the type of analytes and sensitivity/selectivity requirements. For chemicals with a high UV-Vis adsorption, such as pigments, a photodiode array (PDA) detector could be used. This would allow a combination of the LRI identification tool with an UV-Vis spectral library. The use of such a detector automatically excluded the employment of reference compounds that lack a chromophore. It is worth noting that comparable UV spectra are not required for analytes and reference compounds since the use of a PDA allows the

observation of the chromatograms of the unknown sample and the reference series at different wavelengths. What is essential is that they undergo the same elution process from the injection port to the detector. Mass spectrometry is considered a universal detection system; however, some molecules can be difficult to ionize with the ionization source more suitable to the analytes of interest. Within this context, the use of the Single Ion Monitoring (SIM) mode to improve sensitivity for the reference compounds can be advantageous. Finally, detection systems such as a fluorimeter, selective for fluorescent molecules, or a light scattering detector, which is better for large molecules, make finding a suitable homologue series extremely difficult, unless it is possible to use compounds from the same chemical class as the analytes, e.g., triacylglycerols to determine other triacylglycerols. However, it is not common to find homologue series in the real world, such as in pigment or vitamin analyses, as well as pesticides and contaminants.

4) Low cost and widespread market availability. The possibility to synthetize some substances is not a pratical option, as it is both expensive and time-consuming. Furthermore, some laboratories could not have such a possibility.

5) High stability under a wide range of elution conditions, such as different temperatures or mobile phase pH.

6) Low toxicity.

Considering such requirements, it is possible to critically mention the attempts performed over the past years in order to establish the use of RI or LRI in LC. Baker and Ma [12], proposed the use of a ketone standard mixture (i.e., from acetone to 2-tricosanone) as homologous series in isocratic conditions, followed by the RI calculation using the Kovátz logarithmic equation. Despite the lack of chromatograms in the research article, it is acceptable to assume that the choice of twenty-one reference compounds ensures a satisfactory coverage of the chromatographic space. Baker and Ma also compared the RI values obtained on two different RP chromatographic columns based on an octadecyl- and a cyano- bonded silica stationary phase, and investigated at how the RI changed when the mobile phase composition, viz. the organic solvent percentage, was chanced. They immediately pointed out that, among the several compounds examined, the RI remained quite constant only for the acetophenone standard, which, according to the second criteria, is the one closest to the reference ketones in terms of chemical properties. The other compounds showed, in many cases, a decrease in RI when the amount of organic solvent percentage (methanol or acetonitrile) was increased due to their major affinity for these solvents compared to ketones, related to the presence of other polar groups. On the other hand, the ketones show a strong affinity with the stationary phase and are less influenced by the change in the mobile phase composition. However, this reference series was no

longer mentioned in literature, probably due to its low UV absorption and poor applicability in several analytical areas.

Because of its higher UV absorption, three years later, Smith et al. [13] suggested the use of an alkyl aryl ketones homologous series, from acetophenone to heptanophenone. In that and later works [15,19–22,33–35], the influence of the stationary phase chemistry and packaging, as well as the mobile phase composition, was again evaluated, leading to conclude that, due to the occurrence of significant RI deviations even under slightly different analytical conditions (e.g. the lot number of the column), a standardization of LC conditions was necessary to create a reliable identification method and a usable database at inter-laboratory level, that is the first task of the RI theory. Using CO_2 and different percentages of methanol as mobile phases on two different packed columns, the same RI approach was used to determine barbiturates in SFC, and the results were similar [36]. Moreover, the alkyl aryl ketone series lacks the first requirement (see above) since it does not cover the region of the earlier eluted compounds, *viz.* the more polar molecules. In this regard, Bogusz et al. proposed the use of 1-nitroalkanes (i.e., from nitromethane to 1-nitrooctane) as reference homologue compounds in the same years, which showed a better coverage of the chromatographic space under common RP conditions, especially when a gradient elution mode, from 90% to 30% of water (10-70% of acetonitrile), was used [16]. In this case, Eq. (1.4) was used to calculate the LRI because the gradient mode in LC is comparable to a temperature programmed run in GC. The use of a 1-nitroalkane series *vs.* alkyl aryl ketone series for the identification of basic drugs [17] is shown in Fig. 1.4. It is evident that the latter does not cover the region of the most polar molecules, which are eluted with low organic solvent percentages, specifically up to 30-35%, whereas the first alkyl aryl ketone (acetophenone) is eluted around 40% of organic solvent. Extrapolating the retention time of a hypothetical reference compound eluted before the analytes, the LRI for the earlier compounds is determined. Such an extrapolation negatively affects the LRI reproducibility of these compounds, causing misidentification. On the other hand, 1-nitroalkanes cover the entire elution range, avoiding the need for any extrapolation, resulting in good LRI reproducibility in six different octadecylsilica columns (two different brands and three production batch for each brand) [17]. Nevertheless, nitroalkanes are hazardous and carcinogenic (against the sixth requirement), and they are difficult to buy: the last two compounds, namely 1-nitroheptane and 1-nitrooctane, were not commercially available and were obtained by synthesis, in contrast with the fourth requirement.

Furthermore, the further application of the nitroalkane series to additional chemicals reveals limited RI reproducibility, experimented by any chance of the analytical parameters, instrumentation, or even column packaging, thus hampering the widespread use of RI databases at both intra- and inter-laboratory levels [22]. Yamauchi [37] made a similar attempt to establish phenol derivatives as

reference compounds for the analysis of pharmaceutical compounds. Only those phenols that maintained the same elution order under different RP-LC conditions, namely different stationary phase (octadecylsilica, octylsilica, and phenylsilica) and mobile phase composition (different percentages of acetonitrile or methanol in water and different pH), were considered suitable for the RI calculation. Reproducible results were obtained using parabens (alkyl- 4-hydroxybenzoates from methyl- to nonyl-) as reference standard compounds. Over the course of two years, the reproducibility was tested on different columns, mobile phase conditions, and temperature. However, phenols having nitro, halogen or amino substituents showed significant RI variations when the RP-LC conditions were changed, indicating that the second requirement is not respected. Other applications developed during the same years included the determination of mycotoxins and steroids [38–40] employing both the alkyl aryl ketone standard mixture and a novel 1-[p-(2,3- dihydroxypropoxy)phenyl]-1-alkanone reference series. The latter better fitted the analytical purposes because of a total coverage of the more polar region. Even in this case, the RI stability by changing numerous experimental parameters, such as column lot and temperature, focused the research interest. Changing the temperature by more than 20 °C caused a significant RI variation, whereas almost constant values were calculated on different columns (different production lot) [38,45]. However, such a homologue series was no longer reported since single compounds are not commercially available and are complicated to be synthesized (against the fourth requirement).

UV detection was used in all of these applications, and UV spectra were used complementarily with RI during the identification. Only one scientific paper reported the combination of RI with MS data for the study of micotoxins using the 1-[p-(2,3-dihydroxypropoxy)phenyl]-1-alkanone reference series on two different instrumental setup (different columns, ion sources, and mobile phase compositions) [39]. Small differences in the RI values were observed, but only a limited number of substances were studied [41]. In reality, with exception of a few application in 2005 [42,43] and 2009 [44], the RI and LRI approaches have been abandoned for over two decades. M.A. Quilliam suggested a new RI (or LRI) scale for the detection of unknowns in LC [45 ,46] in 2013, following the notable progress in the field of MS and tandem MS detectors. The Quillam's scale was based on 1-alkylpyridinesulfonic acids, which, differently from the three mixtures previously discussed, consisting of small and/or medium polarity compounds, generated a high MS signal under both electrospray (ESI) and atmospheric pressure chemical ionization (APCI) ionization, in both positive and negative ionization modes, related to the presence of both the positively charged pyridinium functional group and the negatively charged sulfonic function. The innovation was tested on a variety of analytes, including drugs, pesticides, toxins, and dyes, in order to establish the extensive applicability of the proposed homologue series, which covers a large range of masses and retention

times, allowing the interpolation of a wide range of compounds. However, no research papers have been discovered in literature dealing with the use of such homologue series for RI calculation, probably due to their unavailability on the market. In addition, the compounds included in the homologue series have still not been studied from a toxicological point of view and could present very high toxicity due to the presence of the pyridine ring.

Mondello et al. suggested a novel LRI approach for identifying TAGs in food and biological samples that uses triacylglycerols (TAGs) containing only odd carbon number saturated fatty acids, from trinonanoin to trinonadecanoin [47,48].

The purpose of this approach is to assume that reference compounds and target analytes have highly similar chemical structures, such that they respond similarly to any variation in operating conditions. A systematic study was carried out to confirm such a hypothesis and evaluate the reproducibility of the LRI. This study was carried out by systematically changing the analytical conditions. The influence of column dimension and stationary phase packaging, flow rate, oven temperature, gradient steepness, and mobile phase composition was considered, highlighting in most cases, a perfect agreement between all the conditions. It was possible to assess that "the LRI database can be used under different conditions provided that the thermodynamic factors, mainly expressed by the partition coefficient, are not considerably altered. Therefore, column dimensions, flow rates and gradient steepness have almost no influence on LRIs, while mobile phase and column temperature need to be fixed to successfully use the LRI identification system" [47]. In terms of column packaging technology, monodisperse sub-2 μm (particle size 1.9 μm), and partially porous (particle size 2.7 and 2.0 μm) columns gave repeatable LRI values, while totally porous (particle size 3 μm) columns gave completely different values, most likely due to a different interaction mediated by larger totally porous particles (particle size >> 2 μm), especially considering the differences kinetic properties between saturated reference TAGs and the analytes, often characterized by a certain degree of unsaturation. This systematic study opens up new perspectives for the application of LRI in LC, since, analysts have a clearer idea about the experimental conditions which need to be standardized, compared to earlier studies. On the other hand, advances in column packaging technology and equipment are paving the way for the LRI approach to be used for routine LC analysis. However, both the previous and the most recent studies have demonstrated the difficulties of creating a universal retention index scale in LC. The reference compounds, in fact, will be chosen based on the application. In recent literature, TAGs were used for lipid analysis and alkyl aryl ketones were used for oxygen heterocyclic compounds with high reproducibility, even when the column and instrumental setup were changed.

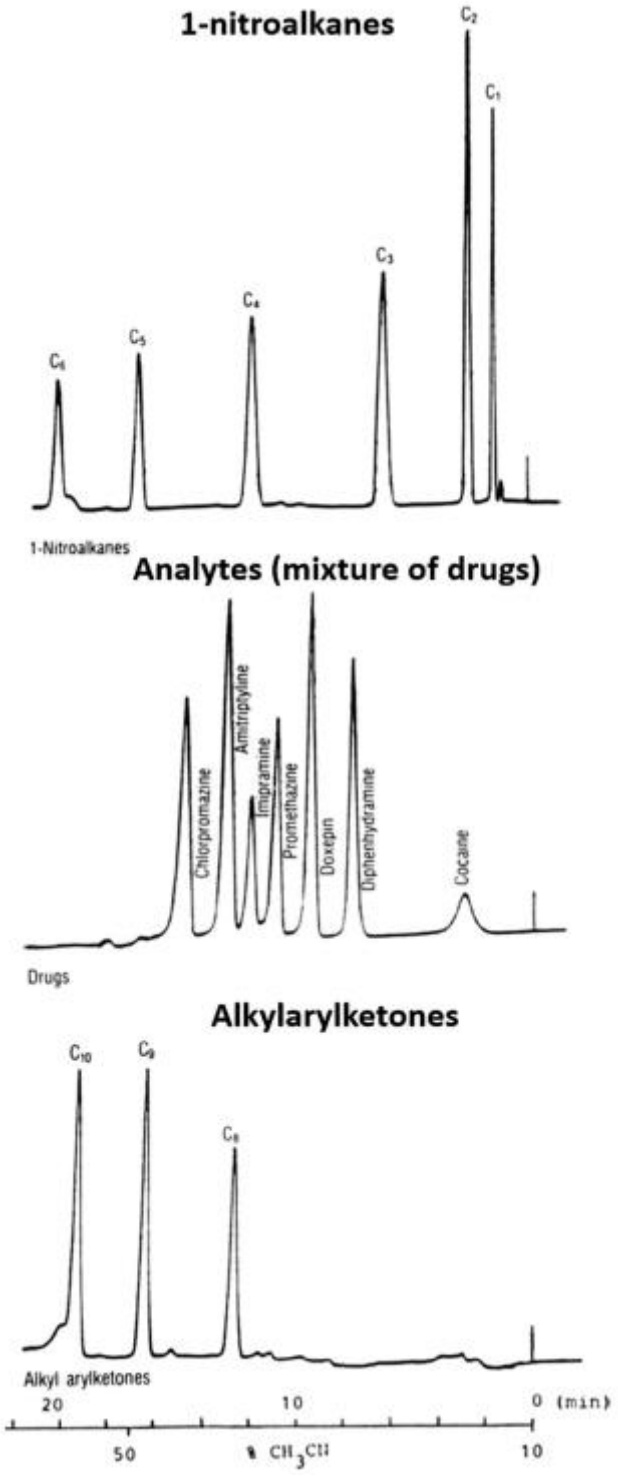

Figure 1.4. *Comparison between 1-nitroalkane and alkyl aryl ketone reference homologue series for the identification of basic drugs.*

1.2.4. Hystorical applications and recent progresses

The use of retention indices in LC during the 1980s and 1990s, often in combination with UV spectral information for the identification of drugs in toxicology research. Table 1.1 shows a complete list of

applications for those years, along with the employed reference series and some key points of the research.

The standardization of LC methods and retention parameters centralized the interest of the researchers, particularly from the late of 1980s to the early 1990s. The majority of applications regarded the toxicological area [14,16–20,22,38,39,49–53], apart from a paper dealing with a food case-study, that is, the determination of curcumin in turmeric [54], an article dealing with a clinical study, reporting the analysis of carotenoids, tocopherols, and retinoids in human serum [55] and two research works that aim to create a RI database for phenolic compounds to be applied in botanics [42,43]. Many research papers also included fundamental studies for the characterization of different stationary phases, such as evaluating the RIs of standard compounds (e.g. toluene, nitrobenzene, p-cresol, and 2-phenylethanol) and their stability under various operating LC conditions [15,21,33,34,44,56,57]. Batch-to-batch reproducibility in column packaging technology has been extensively studied, leading to satisfactory results only from the end of 90's [44,58], related to the tangible developments in modern LC instrumentations and stationary phase production.

Table 1.1. *List of publications dealing with the use of RI in LC, along with the application field, homologue series used, analysed compounds and highlighted aspects in the corresponding references.*

Reference	Year	Application area	Analytes	Homologue series	Key-points
[12]	1979	Pharmaceuticals /Toxicology	Narcotics/Drugs	2-keto alkanes	Two different columns were compared and only compounds structurally similar to homologue compounds had stable RI values
[13]	1982	Fundamentals	Aliphatic ketones and aromatic compounds	Alkylarylketones	Different columns and eluents systems were compared, leading to the conclusion that a standardization of the LC conditions is necessary to reliably apply the RI approach
[33,34]	1982	Fundamentals	Aromatic compounds	Alkylarylketones	
[14]	1984	Toxicology	Mycotoxins	Alkylarylketones	Since a gradient elution was used for mycotoxin separation, the linear equation was applied for the LRI calculation, rather than the logarithmic relationship
[54]	1984	Food	Curcumin	Alkylarylketones	The RI values were used to distinguish between curcumin and minor curcuminoids. Such values in different eluent systems provided useful information about the position of methoxyl substituents on the phenolic rings
[19,20]	1984	Toxicology	Barbiturates	Alkylarylketones	The effect of the stationary phase and the mobile phase was investigated. RIs can be considered stable only for small variations of the operating conditions
[15]	1987	Fundamentals	Aromatic compounds	Alkylarylketones	
[18]	1987	Toxicology	Thiazide diuretics and related drugs	Alkylarylketones	
[52]	1987	Toxicology	Mycotoxins and other secondary fungal metabolites	Alkylarylketones	UV-Vis spectra were used in combination with LRI calculated according to the linear equation (Eq. 2)
[53]	1987	Toxicology	Barbiturates	Alkylarylketones	RI can be very stable by applying a correction factor based on the RI shift of two reference standards, provided that the latter belong to the same chemical family of the analytes (in this case barbiturates)
[16]	1988	Pharmaceutics/ Toxicology	Drugs (several classes)	1-nitroalkanes	It is pointed out that nitroalkanes show a better coverage of the elution range, compared to alkylarylketones

[17]	1988	Toxicology	Basic drugs	Alkylarylketones and 1-nitroalkanes	The 1-nitroalkane scale gave more reproducible LRI values compared to the alkylarylketone one, especially for more polar drugs not covered by the alkylarylketone mixture (see section 2.1)
[59-61]	1989	Fundamentals	Monosubstituted Aromatic compounds	alkylarylketone	A model for RI prediction based on the chemical structure of the analytes was implemented. A correlation between predicted and experimental values exists
[21]	1989	Fundamentals	Aromatic compounds	alkylarylketones	Different stationary phases (cyano and octadecylsilica) and organic solvent percentages were used for the elution of test compounds, leading to the conclusion that a standardization of the LC conditions is necessary to reliably apply the RI approach
[38]	1989	Toxicology	Mycotoxins	1-[*p*-(2,3-dihydropropoxy)phenyl] -1-alkanones	Satisfactory specificity was achieved by combining LRI with UV-Vis spectral data
[39]	1991	Toxicology	Trichothecene (mycotoxins)	1-[*p*-(2,3-dihydropropoxy)phenyl] -1-alkanones	This is the first work in which the RI approach was combined to MS data
[50]	1991	Toxicology	Therapeutic drugs, drugs of abuse, and pesticides (both basic and acidic/neutral)	l-Nitroalkanes	RI were used in combination to UV spectra, generating a database of 225 substances. A real-world sample (human blood) was also analyzed
[22]	1991	Toxicology	Polar drugs	Alkylarylketones, alkan-2-ones and 1-nitroalkanes	The alkylarylketone mixture was the less suitable for the RI calculation of polar compounds. However, a poor reproducibility was achieved by using all the three retention index scales: a standardization of the LC conditions is necessary to reliably apply the RI approach
[49]	1992	Toxicology	Basic drugs	Alkyl aryl ketones	The RI stability of basic compounds at different pH depends on the pKa of each compound, related to its the ionization degree which affect the interaction with the mobile phase
[36]	1993	Fundamentals	Phenols	n-alkyl-4-hydroxybenzoate (parabens)	Parabens were selected as they showed a stable elution profile under different operating conditions. A prediction model was also developed to predict the retention of analytes under several experimental conditions
[53]	1993	Toxicology	Basic, acidic and neutral drugs	1-nitroalkanes	An interlaboratory RI comparison was carried out, achieving a precision of ±10 units, by applying a correction factor based on the RI shift of two reference standards, as in previous works [46]
[55]	1993	Food/clinical	carotenoids, retinoids and tocopherols	Alkylaylketones	Bioactive molecules were analyzed in human serum and in the sera and livers of mice that had received supplements of retinyl acetate
[58]	1996	Forensic/ Toxicology	Drugs	1 -nitroalkanes	An interlaboratory study was carried out, by comparing three different databases. A good agreement was observed when the same LC conditions are employed, even by changing the column lot, while the mobile phase conditions (pH and organic solvent) need to be standardized

[40]	1998	Toxicology/clinical	Steroids	1-[*p*-(2,3-dihydropropoxy)phenyl]-1-alkanones	Steroids were determined in serum samples by using LRI in combination with UV data
[42]	2005	Botanics	Phenolic Compounds	Alkylarylketones	An algorithm for the extrapolation of RI for hydrophilic organic compounds eluted before the first reference component (acetophenone) was developed, which provided reproducible results at least at intra-laboratory levels
[43]	2005	Botanics	Phenolic Compounds	Alkylarylketones	A certain correlation between RI and the functional groups on the phenolic ring was established
[44]	2009	Fundamentals	Aromatic compounds	Alkan-2-ones and alkylarylketones	The RI stability by changing different operating conditions was evaluated by using different calculation methods and homologue series. The best results were achieved by using the alkane-2-one scale.
[45,46]	2013	Food/Toxicology/ Forensics/ Pharmaceutical	Toxins (e.g., biotoxins or other poisons), pharmaceuticals, drugs of abuse, peptides, persistent environmental pollutants, food contaminants	1-alkylpyridinesulfonic acids	This new homologue series was introduced, since it provides a good MS response, under both positive and negative ionization modes, by using both ESI and APCI interfaces for LC
[47,48]	2018	Food	Non-polar lipids	Odd carbon number triacylglycerols	A systematic study changing one by one the analytical conditions was performed by establishing which parameter need to be fixed for the universal use of the LRI system. Flow rate, column dimension and packaging technology, gradient steepness, connection and detection system, and small temperature variations have no influence on the calculated LRI. This positive result was mainly to the quite identical chemical structure of analytes and reference compounds
[62]	2018	Food	Non-polar lipids	Odd carbon number triacylglycerols	The application to the hydrolysis products of vegetable oils allows to add in the database mono- and diacylglycerols
[64]	2019	Food	Non-polar lipids	Odd carbon number triacylglycerols	The LRI approach was applied to the lipid extract of pistachio nuts.
[66]	2019	Food/cosmetics	Oxygen heterocyclic compounds	Alkylarylketones	The reproposal of the alkylarylketone homologue series was based on the higher batch-to-batch reproducibility in instrumentation and column technology. LRI calculated from two different instrumental setup provided reproducible results.

[67]	2019	Food/cosmetics	Oxygen heterocyclic compounds	Alkylarylketones	The combination with commercial EI-MS spectral libraries led to a reliable, fast and automatic univocal identification of each compound.
[63]	2020	Food	Non-polar lipids	Odd carbon number triacylglycerols	The combination with MS data was mandatory, given the high complexity of fish samples
[65]	2020	Food	Non-polar lipids	Odd carbon number triacylglycerols	A new software was implemented to automatically identify lipids in fish samples based on the LRI database. The combination with MS data was mandatory, given the high complexity of fish samples
In preparation	2020	Food/cosmetics	Oxygen heterocyclic compounds	Alkylarylketones	The application of the LRI filter allows to discriminate between isomer characterized by the same MS/MS fragments

In order to implement the RI database, some prediction models were also created [37,59–61]. The presence of specific functional groups, as well as a correlation with chemical structure, were used to predict retention parameters.

As previously mentioned, the RI scale based on odd carbon number TAGs for non-polar lipid identification , introduced in 2018, has brought strength and confidence to the stable use of LRI in lipidomics [47,48]. A mixture of saturated TAGs from C9C9C9 to C19C19C19 is normally used as a reference homologue series and the general Eq. (1.4) is chanced as follows (Eq. (1.5)) for LRI calculation:

$$LRI = 100\left[z + 6\frac{t_{Ri} - t_{Rz}}{t_{R(z+6)} - t_{Rz}}\right] \qquad [Eq.\,1.5]$$

where z is the equivalent carbon number (ECN) or partition number (PN) of the reference TAG eluted immediately before the target compound. Since it is related to the carbon chain length CN and double bond number DB (ECN = PN = CN-2DB) of the three fatty acids bound to the glycerol backbone, z spans from 27 for C9C9C9 to 57 for C19C19C19, and 6 is the difference in z units between adjacent reference TAGs. The first research reports a LRI database includes more than 200 TAGs, identified in different foodstuffs [47]. The separation achieved for the most complex analyses samples within three different types of matrix is shown in Fig. 1.5: borage oil (Fig. 1.5 A), goat milk (Fig. 1.5 B) and menhaden oil (Fig. 1.5 C) among vegetable oils, dairy products and fish samples, respectively. The chromatogram of the new reference homologue series is also included (Fig. 1.5 D) to show how it completely covers the elution range of target analytes in various matrices, ranging from vegetables with highly saturated and long-chain fatty acids mixed into high PN TAGs to milk samples with short-chain fatty acids contained into low PN TAGs.

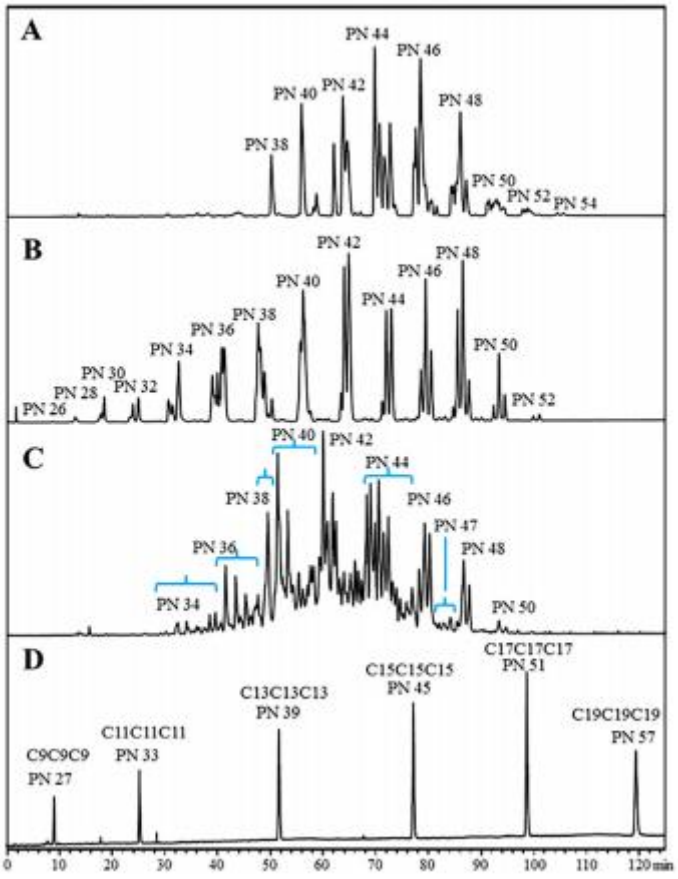

Figure 1.5. *RP-UHPLC profiles of: (A) borage oil, (B) goat milk, (C) menhaden oil, and (D) reference homologue series composed by odd carbon number saturated TAGs from C9C9C9 to C19C19C19*

Furthermore, the separation between compounds characterized by the same carbon number (CN) and double bonds (DBs), i.e. the same molecular weight, is improved thanks to the use of a high-resolution LC method, related to the serial coupling of two sub-2 μm narrow-bore columns, resulting in a difference in LRI values higher than 15 units, ensuring a reliable identification, also according to theoretical calculations. As shown in Table 1.1., this technique was effectively used to analysis of food samples in the years 2018 to 2019, starting from simple vegetable oils and progressing to more complex fish samples, passing through the lipid extract of pistachio kernels. The LRI filter was used as a stand-alone tool to achieve a univocal identification of single components, while in others it was required to combine with MS data to select the right candidate. The first application regarded the identification of the lipid profile of vegetable oils before and after enzymatic lipolysis [62]. The next step was performed in order to obtain mono- and diacylglycerols (MAGs and DAGs) to be used as emulsifiers in the food industry. There are a total of 25 lipids included in the identification table for that work, including three pairs of acylglycerols characterized by the same CN and DBs, namely dilinolein and linolenoyl-oleyl-glycerol (CN 36 and DBs 4), trilinolein and linolenoyl-linoleoyl-

oleoyl-glycerol (CN 54 and DBs 6), dilinoleoyl-oleoyl-glycerol and dioleoyl-linolenoyl-glycerol (CN 54 and DBs 5), perfectly distinguished each other thanks to an LRI difference of about 20 units. From an analytical point of view, the study allowed for the inclusion of additional MAGs and DAGs into the LRI database, which are normally not present in real-world samples. In this regard, any sample is considered a valuable source of a new lipid species for costant database implementation, provided it is validated by the MS spectrum. As a results, two years later, a similar study was conducted on a fish oil, specifically a *Musteleus mustelus* liver oil, and on the hydrolysis products obtained by the action of two different lipases [63]. A total of 317 acylglycerols were identified, including MAGs, DAGs, and TAGs, many of them not originally present in the database. Meanwhile, the LRI database was utilized to confirm the identification of pistachio lipids, which had been identified using a new shotgun-MS system equipped with a tandem high-resolution MS analyzer (quadrupole–time of flight) that does not allow for any isobaric compounds discrimination [64]. Within this context, the need of a chromatographic separation, which provides retention data, was assessed.

Finally, the novel approach was used for the analysis of sea bass samples, which led to the identification of 70 compounds [65]. In that study, the total fatty acid profile obtained by GC methods after conversion of intact lipids into more volatile and less polar fatty acid methyl esters also supported the identification of TAGs, excluding from the LRI search TAGs composed of fatty acids not detected (or detected at a percentage lower than 0.3%) by GC. This allows to significantly reduce the list of candidates arising from the LRI search by automatically creating a restricted database depending on the GC results, which are obtained faster and easier with respect to LC-MS. As an example, in Fig. 1.6, a software developed ad-hoc for this type of applications, reports the automatic identification of an unknown peak. The figure shows that, by using the total LRI database of more than 200 TAGs, a list of 8 candidates was obtained, while by considering the fatty acid profile coming from GC-MS analysis, the list was reduced to only 3 candidates, due to the absence or very low content of some fatty acids in the analysed sample, namely gamma linolenic acid (γLn), myristic acid (M), lauric acid (La) and capric acid (Ca). Going into details, the restricted LRI database alone allowed to unambiguously identify more than 1/3 of peaks.

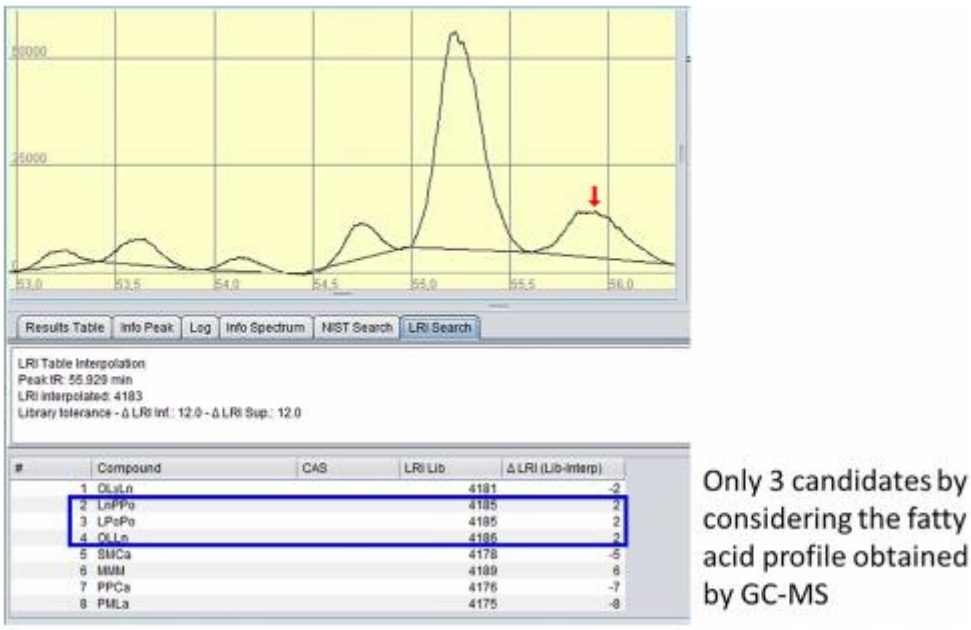

Figure 1.6. *Automatic identification of an unknown, by match with the total LRI database of TAGs and selection of a reduced list of candidates (in the blue box) by using a restricted database according to the fatty acid profile coming from a GC-MS analysis.*

Theoretically, the adopted approach could be realized by using basic LC instrumental setups, such as an LC coupled to an evaporative light scattering detector, without the need of sophisticated detection systems, i.e. MS. Additionally, when MS is used, the LRI identification tool reduces the tedious and time-consuming manual interpretation of the MS spectrum under each peak to a confirmation of the MS fragments expected for each candidate of the LRI search. In other words, although several adducts may be present in an LC-MS spectrum, the LRI filter considerably restricts the list of possible TAGs for each molecule related ion detected in the MS spectrum. At the moment, the LRI approach is mainly used to analyze food products. Its clinical use, e.g., to the analysis of biological fluids and tissues is extremely attractive, especially given the complexity of such samples. The coexistence of several lipid classes, many more numerous compared to foodstuffs, contributes to their complexity. For example, several phospholipid classes are commonly found in a clinical sample, and their separation requires the use of a more polar mobile phase, such as water, to properly elute and resolve all the classes. Future research in the fields of lipidomics could focus on the extension to polar lipids. The use of the LRI strategy to analyze the non-volatile fraction of citrus essential oils, in particular for the determination of oxygen heterocyclic compounds (OHCs), implied the reutilization of the most historically employed alkyl aryl ketone reference homologue series. In fact, the polarity range of OHCs, which are classed as coumarins, furocoumarins and polymethoxyflavones, is well covered by alkyl aryl ketones. Initially, the LRI filter was combined with an UV-Vis library [66], as in previous applications (Table 1.1.), although this method has never been used for the identification of

OHCs in the past years. In this case, LRIs were calculated according to Eq. (1.2) and were helpful to discriminate between isomeric compounds with the same UV-Vis spectra. Most likely, they will be characterized by identical MS spectra, so their discrimination according to the LRI looks very promising for their univocal identification. Subsequently, seven citrus essential oils were injected into a nano LC-EI-MS prototype to prove this hypothesis. For the first time in LC [67], LRI and EI-MS spectra were combined in a dual-filter spectral library. The use of EI-MS detection in LC was made possible by miniaturizing the LC flow to liquid amount compatible with the high vacuum system of the EI source. The commercial EI-MS library was used in this prototype system to produce a rapid and reliable identification. Furthermore, the generated spectra and the chromatograms were processed by the same software used in GC-MS, which allowed for the first time in an LC-MS platform to achieve the same identification procedure of a GC-MS analysis. Fig. 1.7 A shows the spectral similarity results for furocoumarin imperatorin against the commercial NIST (National Institute of Standards and Technology) EI-MS library. Using only the MS identification filter, the compound could be classified as imperatorin and its isomer isoimperatorin with spectral similarities greater than 90%. The application of the additional LRI filter automatically excluded the latter because of a difference between calculated and tabulated values larger than the maximum tolerance of 4 units (Fig. 1.7 B), established depending on theoretical calculations [67]. As a result, by setting the maximum tolerance of ±4 LRI units, Fig. 1.7 C reports the univocal identification of imperatorin. Other isomeric structures, meanwhile, were correctly identified without any mismatching due to a difference in LRI units that much exceeded the maximum variability for each LRI value. Hexamethoxyflavone and nobiletin, as well as 8-geranyloxypsoralen and bergamottin, are completely resolved in the chromatograms with LRI differences of 49 and 74 units, respectively [67].

Finally, the nano LC-EI-MS system could be a useful tool for the simultaneous application of LRI and MS identification criteria. However, due to the intrinsic limitations of EI source, viz. difficult vaporization and ionization of this kind of molecules, this prototype has poor applicability for large biomolecules. As a results, in many analytical fields, the implementation of a comprehensive analytical platform able to rapidly, reliably and automatically identify large molecules based on the retention behaviour combined to spectral information is of great importance.

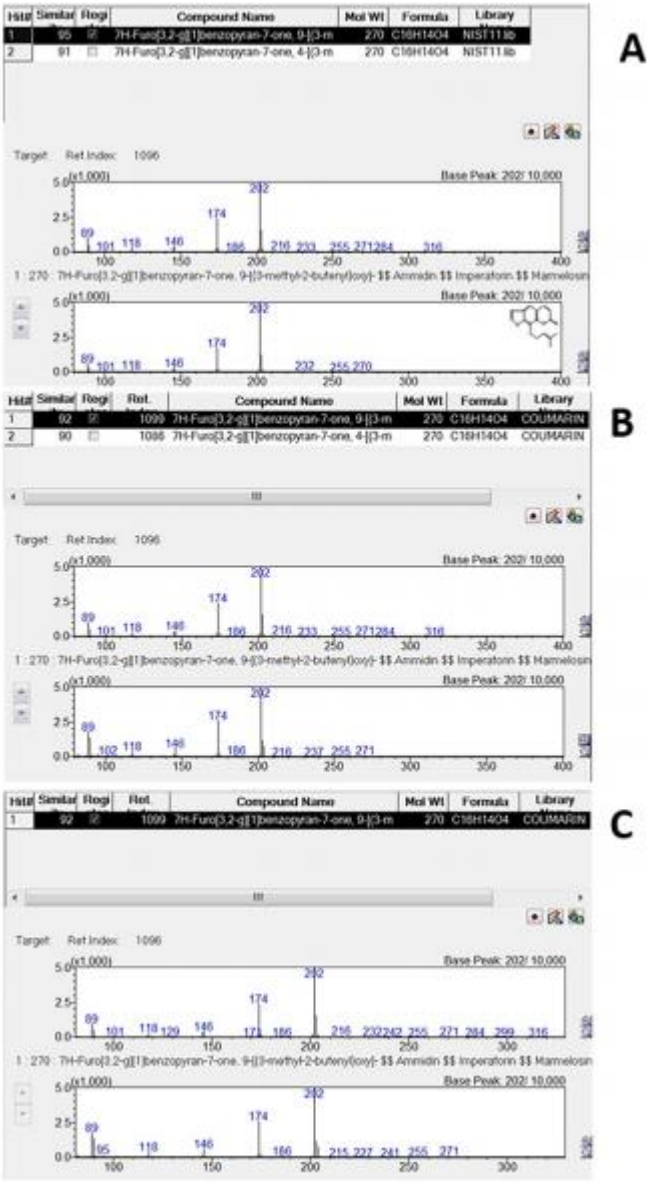

Figure 1.7. *(A) Identification of imperatorin by using the commercial NIST EI-MS library. (B) Identification of imperatorin by using a lab-made EI-MS library with embedded LRI values. (C) Univocal identification of imperatorin by applying simultaneously the EI-MS (similarity major than 90%) and LRI (tolerance of ± 4 units) filters.*

1.2.5. Need of a dedicated software

The lack of a commercially available software able to handle on LRI database and performing automatic identification of an LC chromatogram using the LRI approach has prompted the development of a new ad-hoc software for the use of the LRI filter in LC. Common LC data processing software usually allows an automatic peak assignment only based on retention time or relative retention time, with no assurance of applicability over time or at inter laboratory levels. According to the authors' experience, some software has the LRI function, which allows the LRI calculation on the basis of a reference series previously integrated and loaded into a dedicated

window. The correspondence with a database, on the other hand, must be done manually through a visual comparison between tabulated and experimental values, which takes a lot of time and effort. Only recently, using commercial software for GC-MS as an example, has novel software been developed for the importation of LC data coming from different instrumental setups (different instrumental brand and different kind of detectors) [65]. The software combines the LRI database search with a spectral match against a MS or UV library depending on the detection system. The use of two identification filters, namely the LRI and the spectral information, in a complementary way, made the identification procedure as similar as possible to the well-established process in GC-MS: compounds having similar spectral properties are discriminated against on the basis of different LRI values, by automatically excluding compounds not falling in the LRI window determined by the maximum tolerance on each LRI value; on the other hand, compounds characterized by similar retention behavior are distinguished by different spectral data. One to the lack of MS spectral libraries, the program has has so far only used the LRI filter to obtain an univocal identification, despite being able to perform a dual-filter search. In particular, the software operates as follows: 1) peak integration; 2) LRI calculation according to Eq. (1.4), by using a reference homologue series previously integrated; 3) search in the LRI database, consisting of an Excel file readily converted by the software in the suitable format; 4) visualization of the MS spectrum, if available (in the case of HPLC-MS analyses); 5) selection of the right candidate (one or more) on the basis of the MS spectrum (if available), literature information or data coming from complementary techniques; 6) data reporting, which provides the list of identified compounds, along with the comparison between tabulated and experimental LRI.

For clarification, Fig. 1.8 shows an example of the univocal identification by the new software of the TAG DhOP (docosahexanoyl-oleyl-palmitoyl-glycerol) in a sea bass sample. The figure also shows the screenshot of the novel software containing the following panels: a) entire or zoomed chromatogram on the top left panel; b) qualitative parameters on the top right panel; c) qualitative results on the bottom panel, reporting the list of candidates for the selected peak along with the calculated LRI, the tabulated value and their difference. The top right panel is focused on the LRI calculation conditions: the previously integrated chromatogram of the reference homologue series must be loaded and a table with the retention time of the reference compounds is automatically filled with the LRI values assigned to each reference compound, by setting in a sub-window the carbon number (z in Eq. (1.4)) of the first reference compound and the n value to be applied in Eq. (1.4); in the same window, the LRI database will be loaded. The list of candidates in the bottom panel will depend on the maximum accepted tolerance (Δ), equal to 12 in Fig. 1.8. Such a value derives from

theoretical aspects, namely the calculation of the separation number (SN) and the minimum LRI difference (δ) between resolved peaks able to guarantee unambiguous identification.

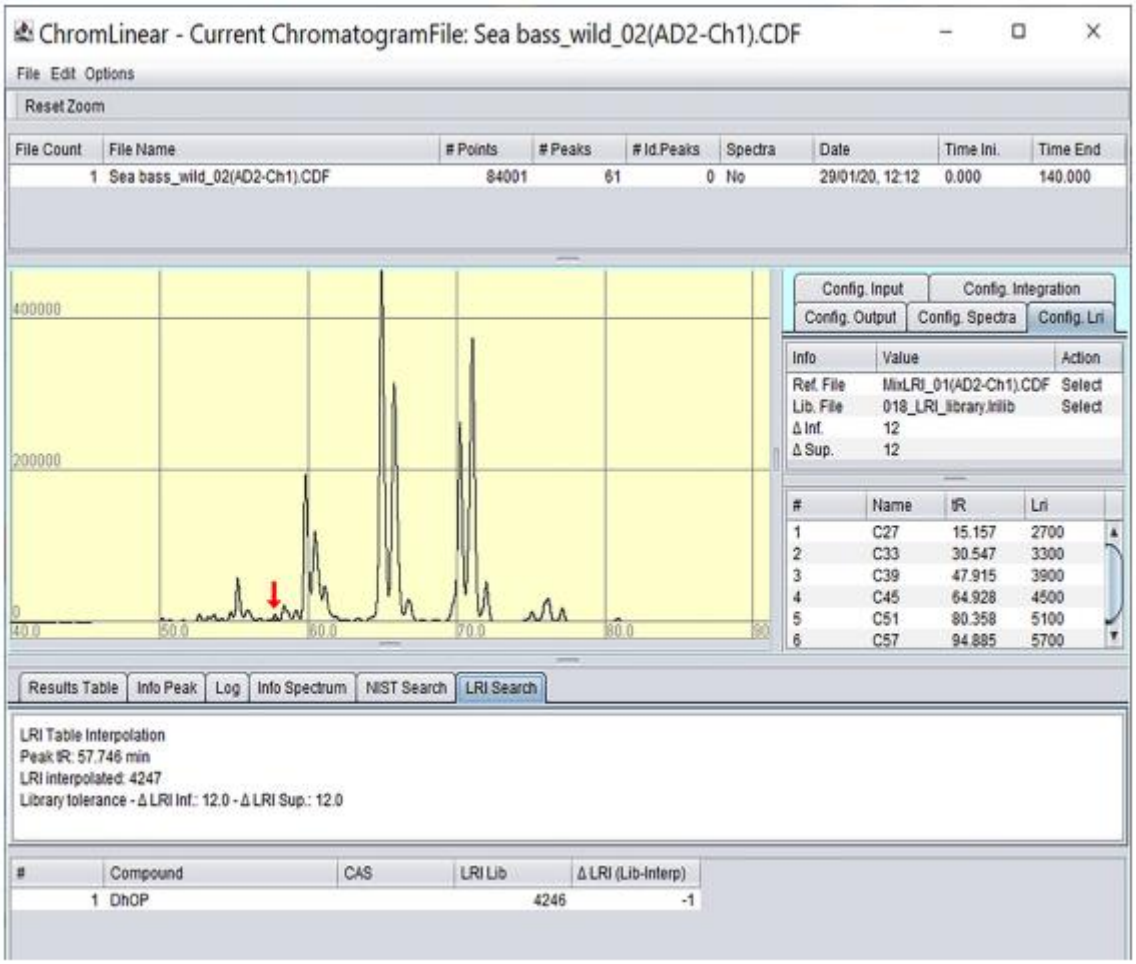

Figure 1.8. *Univocal and automatic identification of a triacylglycerol by using a new dedicated software.*

SN, which represents the number of baseline resolved peaks in a specific time region, is calculated using Eq. (1.6):

$$SN = \frac{t_{R(z+n)} - t_{Rz}}{w_{h(z+n)} + w_{hz}} - 1 \qquad [Eq.\,1.6]$$

where $t_{R(z+n)} - t_{Rz}$ is the retention time difference between adjacent reference standards, and w_{hz} is the width at half height of the homologue peaks. Then, δ is equal to the retention index difference between adjacent reference standards (ΔLRI) divided by SN + 1 and the maximum tolerance, viz. the shift between calculated and tabulated LRI, to be set into the software for the automatic identification, corresponds to δ/2. The chromatogram separation of the reference odd carbon chain TAGs composing the new homologue series applied in lipidomics is shown in Fig. 1.9 A, along with the LRI value for each peak, arbitrarily assigned on the basis of the carbon number, the SN and δ values between

adjacent peaks. In this case, being ΔLRI equal to 600, δ will be 600 / (SN + 1) and spans from 20 to 35. The example of two baseline resolved unknown peaks eluted between the central pair (where δ= 23) in shown Fig. 1.9 B. Each LRI is tabulated with an experimental error, which must be small enough to avoid any overlapping with the adjacent peak. From a mathematical point of view, this error must always be lower than half of δ. As a consequence, δ/2 corresponds to the maximum acceptable tolerance for each LRI during the identification process. In Fig. 1.9, a tolerance of 10-17.5 LRI units will avoid any peak mismatching along the entire chromatogram.

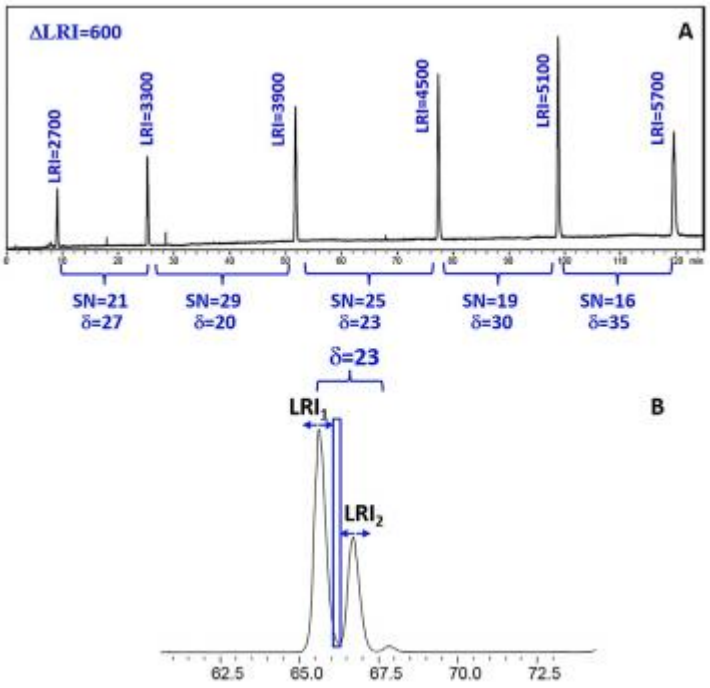

Figure 1.9. *(A) Example of a homologue series, along with SN and δ values; (B) example of baseline resolved peaks.*

If the identification procedure is performed automatically, only one value should be set. Hence, a value of 12 was chosen in Fig. 1.8. In this regard, another criteria of the ideal homologue series may be that homologue peaks in the chromatogram be sufficiently equidistant in the chromatogram in order to ensure identical SN and δ between homologue pairs. Such a requirement allows a unique LRI tolerance (δ/2) to be fixed during the entire chromatographic separation. If δ/2 values are too different between different homologue pairs, spanning from very low values (e.g. 5-10 units) to higher values (e.g > 20 units), the setting of the first could exclude the right candidate for some peaks, while the setting of the latter will surely include wrong candidates for other peaks.

Differently from the example in Fig. 1.8, in other cases of the same research work, a list of several candidates was generated by the software and the elucidation of MS fragments was needed to select the right candidate. Within this context, the future building of the MS library will enable dual-filter search. However, it is necessary to point out that, differently from the LRI database, the MS library

could not be used at inter-laboratory levels, or on changing instrument brand or operating conditions, since API-MS spectra are strictly dependent on the ambient conditions.

1.2.6. Conclusion

This paper discusses advanced developments in the creation of an LRI approach in LC, as well as its application. It was specifically mentioned that unlike GC, universal reference homologous series cannot be used due to the wide range of chemical and physical characteristics, which results in significantly varying chromatographic behaviors. Hence, the reference compounds should be chosen based on the target analytes, in order to ensure that both chemical and physical interaction with the chromatographic system change in a similar way by changing the operating conditions. Future perspectives of LRI in LC could include the application to other analytes, such as polar lipids in the lipidomics area or polyphenols by using the alkyl aryl ketone standard mixture. In the latter field, the use of LRI could be extremely useful, considering, as in lipidomics, the huge number of isomer compounds deriving from the different positions of the substituents (sugar portions, oxydryl group, etc.) on the phenol ring. In the vast majority of cases, their MS and MS/MS spectra are almost similar and MS n experiments will be necessary for structure elucidation, involving a remarkable increase in costs, apart from a major level of MS experience of the researcher. The stability of the LRI could also be tested using different RP-LC stationary phase (C30, C18, C8, Phenyl-hexyl, etc.). The composition of the mobile phase, has already been shown to have a considerable impact on LRI values. However, a relationship between the values obtained under various conditions could be proposed. For instance, the LRI could be plotted against the water % added in A to slow down the separation for more polar species. Additional experiments will be necessary for such evaluations.

[†]This section has been adapted from the following publication: F. Rigano, A. Arigò, M. Oteri, R. La Tella, P. Dugo, L. Mondello in "*The retention index approach in liquid chromatography: An historical review and recent advances*" Chrom. A J. 1640 (2021) 461963.

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Chapter 2

Nano Liquid Chromatography

2.1. Introduction

The particle size reduction, in a fixed-length column, determines the reduction of the dispersion effects, increasing the efficiency and, consequently, the resolution.

In particular Huber, Van Deemter and Kirkland introduced a brilliant idea in analytical chemistry: microparticles with a diameter of 3-5 and 10 μm [1-4].

Over the years, interest in miniaturized techniques has increased enormously, thanks to the numerous advantages offered in terms of operating economy, sensitivity, efficiency, as well as the possibility of an LC-MS interface.

The first studies of small diameter packed columns were conducted by Horvath. The breakthrough came in the late 1960s, when Horvath developed a support with an impenetrable solid core of glass beads (37-50 μm in diameter) coated with an outer layer of porous solid (1-2μm thick). Horvath and collaborators have shown that the porous layer limits the diffusion of molecules into the deep pores, and this leads to an increase in efficiency [5].

The experiments, therefore, were conducted on packed stainless-steel capillary columns, with an internal diameter of 1 mm. Over time they have been replaced by thinner and shorter columns, up to the first fused silica capillaries (20-50 μm ID), introduced by Kennedy and Jorgenson in 1989 [6].

Several research groups have contributed to the development of miniaturized techniques, studying the theory and instrumentation. Nano LC has established itself as a complementary and / or alternative technique to conventional LC [7], and can be used in several application fields such as the pharmaceutical, biological, food, forensic, environmental.

The separation techniques carried out on capillary columns with an internal diameter between 10 and 100 μm are called "*Nano LC*", the separation techniques carried out on capillary columns with an internal diameter between 100 and 500 μm are called "*CLC*".

Generally, the capillary columns used in Nano LC are in fused silica with particle diameter between 3 and 5 μm; the use of the pre-column is recommended to prevent plugging of the column, thus increasing column life, in particular in real-world sample analysis [8].

The advantages of this technique are:

- very low volumes of mobile phase, stationary phase and samples, consequently reduced waste volumes and lower disposal costs;
- reduced environmental impact;
- higher performance
- compatibility with mass spectrometers

For all these reasons such miniaturized techniques still centralize many efforts from both researcher and industrial companies in order to optimize routine analytical methods and to produce robust and

competitive instrumental setup to place on the market. The following sections contain a description of theoretical aspects, of nanoLC components, their historical development, the main drawbacks and troubleshooting.

2.2. Theoretical aspects of Nano-LC

The miniaturized system is a great alternative to conventional LC. All samples analyzed by conventional LC can be analyzed by a Nano-LC, with many positive aspects in accordance with the principles of green chemistry.

Although there are many advantages to using miniaturized systems, several theoretical aspects must be considered.

Reducing internal diameter of the column, the results is a less chromatography dilution with a consequent increase in the concentration of the eluted peak and therefore in the sensitivity [9].

This dilution event, called chromatographic dilution (*D*), is expressed by

$$D = \frac{C_0}{C_{max}} = \frac{\pi d_c^2 \, \varepsilon (1+k)\sqrt{2LH\pi}}{4V_{inj}} \qquad [Eq.\,2.1]$$

where C_o is the initial concentration and C_{max} is the final concentration of the analyte during the chromatographic process, d_c is the column internal diameter, ε is the total porosity of the column, L is the column length, V_{inj} is the sample injection volume and k and H are the retention factor and plate height, respectively.

At the same time, reducing the internal diameter of the column results in a reduction in flow rate and this could lead to an increase in gradient length and lower sample throughput.

In the following section the influence of the different parameters is discussed.

2.3. Instumentation

In Nano-LC the instrumentation is all miniaturized: pumps, injectors, columns, connection system and detector interface.

A more detailed described of these parameters will be presented in this section.

2.3.1. Nano-pumps

Nano-LC pumps must allow reproducible nano flow rates and permit gradient elution at nano-scale levels. Two system can be used in Nano-LC for achieving such features: split and splitless system [9].

The first-generation nano-LC system were based on conventional pumps equipped with a split system. The splitter divides the high flow of the pumps between the nano-column and the restrictor. However, split systems may vary the split ratios of the nano flow, particularly during gradient elution due to the different viscosities of the mixed solvents, decreasing the repeatability of the chromatographic separation [10]. To overcome these problems, splitless system has been development, by using continuous flow system else solvent refill systems. In the solvent refill system the pumps (syringe- or pneumatically driven) force the mobile phase from a loop and have a valve to switch between aspiration and release phases. As a consequence, the flow through the column is stopped during the aspiration phase [11]. Continuous flow pumps are similar to conventional reciprocating pumps with two pistons per channel; they generate a very high reproducibility flow rate, being absent any splitting device.

2.3.2. Nano-tubing connections

Working with very low flow rates implies the miniaturization of each component of the Nano-LC system in order to minimize the void volume that is the total volume of the system starting from the pump up to detector.

Peak broadening is described as a function of the i.d. and length of the capillary [9].

The weaknesses of the Nano-LC technique are the pre-column and post-column dead volumes which can cause the band broadening.

For good chromatographic separation and acceptable analysis time the connection number has to be reduced to minimum, the small tubing and zero-dead volume fittings have to be employed [12].

The Aris-Taylor equation [13] describes the dispersion (σ^2) of solute bands in cylindrical tubes as follow:

$$\sigma^2 = \frac{\pi d^4 L u}{96 D_M} \qquad [Eq.\ 2.2]$$

where d is the ID, and L is the length of the capillary; D_M is the molecular diffusion coefficient; and u is the linear velocity of the mobile phase. Consequently, connection tubing should be kept as short and especially as narrow as possible, often causing an unacceptable increase in backpressure. Generally, for standard nanoLC operations (using 75 μm ID columns and flow rates of approximately 300 nL/min) to overcome issues such as band broadening, delay and high backpressure, a 10-20 μm ID is used pre- and post-column.

Regarding the material, fused-silica capillaries have many advantages over stainless steel or polyether ether ketone (PEEK) tubing, such as: they can be easily cut to the desired length and are readily available in a range of compatible IDs (5-100 μm) with a lower tendency to clog.

2.3.3. Nano-injectors

Generally, the maximum injection volumes for nano-columns is a few nanoliters and is a function of the column length (L), plate number (N), retention factor (k), column ID (d), particles diameter (dp) and some other parameters related to the injection profile (K that is related to the injection profile, by considering normally a tailing effect, and θ that is the fractional loss of the column plate number caused by the injection) [9].

Small injection volumes cause loss of detectability, but larger injection volume produces a broadening of the band and a decrease in separation efficiency.

However, Heron et al. demonstrated that in order to avoid the band broadening effect, it is necessary to use a weaker mobile phase solvent then the sample; this determines an enrichment effect of the analytes on top of the column with a consequent increase in efficiency and sensitivity [9]. In any case large injection volume are still in the range 10-100 nL, not compatible with commercial autosamplers, which usually work at microliter levels and require an instrument adaptation for use in the nanoliter range. To overcome this problem, a split valve between the injector and the column can be used. Alternatively, to direct injection and on-column focusing procedure, it is normally suggested to preconcentrate the sample before the introduction into the nano-column, either off-line, by simple evaporation, or on-line, by using a trap column. Two pumps are used in trapping configuration: a loading pump and a nano-pump, with the trap column installed on a switching valve (Figure 3.1). In such preconcentration setups, relatively large volumes (up to 100 μL) are injected on the trap column using the loading pump at a relatively high flow rate compared to the separation flow. During sample loading, the loading flow is directed to waste; washing salts and other hydrophilic compounds are thus removed, while the nano-pump is directly in line with the nano-column. Once has been the valve switched, the trap column is placed in line with the nano-flow to elute the analytes from the trap

column and separate them on the separation column. In addition, by means of trap columns, the separation column is preserved from clogging.

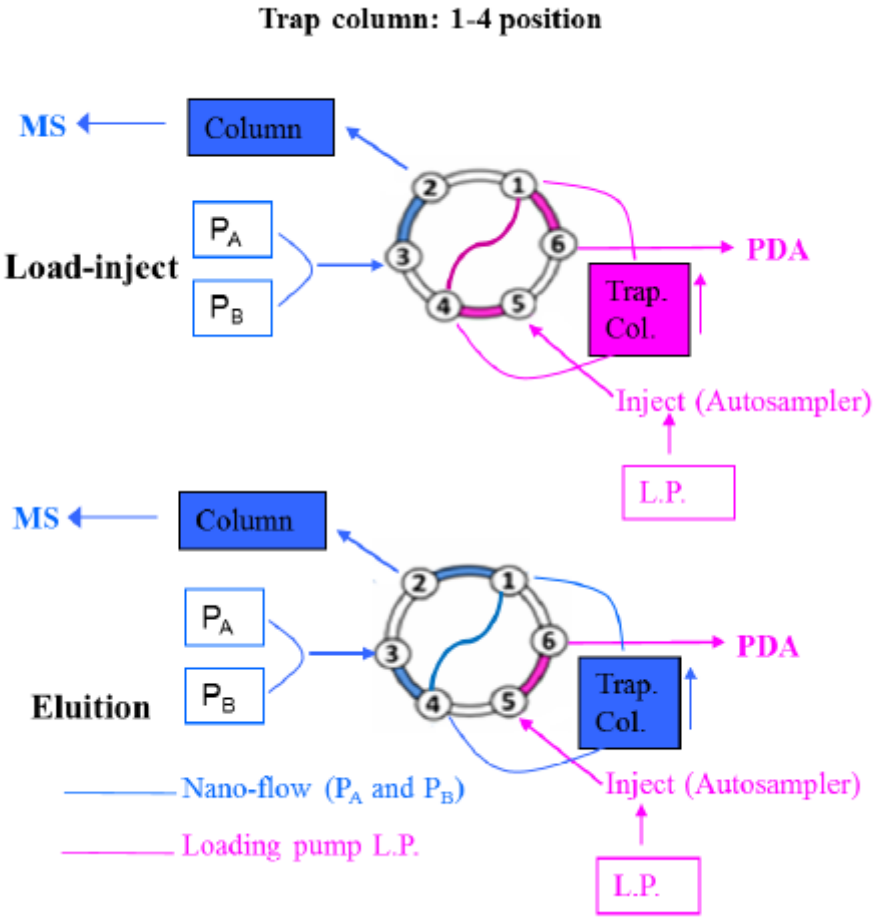

Figure 2.1. *Scheme of the trapping configuration.*

2.3.4. Nano-columns

In general, standard internal diameter for Nano-LC columns is 75 µm; in fact, they represent a good compromise between detectability, loadability and robustness in miniaturized techniques.

Stainless steel and titanium tubes are used for nano-columns, but polyimide-coated fused capillaries are preferred.

Among packed Nano-LC columns, the most common are characterized by 3 µm particle diameter. More recently, particles of 1.5-1.9 µm were introduced and Nano-UHPLC were developed reducing analysis time to 1-4 min [14].

The packing of 20-200 µm ID fused silica capillaries and the preparation of robust frits are not easy. To overcome these drawbacks, the use of tapered capillaries [15-16] and monolithic frits have been proposed [17-18].

Monolithic columns offer some advantages over packed columns, mainly because of the absence of frits. They also have a major loading capacity and a minor clogging probability, thus a longer lifetime. Within this context, in a packed column the presence of even submicroscopic solids in a sample may effectively, and sometimes definitively, clog the column. In fact, there is no possibility to backflush the column as the stationary phase is not protected (no frit) from the inlet side, due to the particular packing procedure of the nano-capillary. Furthermore, too high flow rate and, as an effect, too high pressure on the column often leads to the terminator expulsion, which destroys a column. Again, monolithic columns are more resistant as the overall pressure is significantly lower [19].

2.4. Detectors

The most common detection system for nano-LC are the same as those employed for HPLC separation.

UV-vis photometric detector is very common in Nano-LC, and the UV nano-cell consists of a fused silica capillary tubing with a volume that not exceeding 20 nL.

Mass detection is widespread in biomedical and pharmaceutical applications, thanks to very good level of sensitivity and also because MS detection is universal tool.

Using the nanospray interface we have to pay attention to the flow rate. In particular, applying a flow range of 100-500 nL / min can be considered a good choice since this interface requires only a small amount of eluent from the column [9].

Electrospray Ionization (ESI) is the most common interface used in Nano-LC-MS.

The qualitative and quantitative characteristic and stability of spraying depends on several conditions: an aperture of spraying needle, an applied voltage, a flow rate, and solvent composition [20].

The introduction of a downscaled electrospray ion source that works at a flow rate of 25 nl / min and generates smaller droplets (<200 nm) and the suppression of analyte, pratically absent at flow rates below 20 nl / min, increase the sensitivity of detection compared to conventional ESI [20].

The simplest and most economic method of applying voltage is through a liquid junction in which an electrode makes contact with the mobile phase using a T-piece. Gold fused-silica emitters is a good alternative, but the metal coatings can deteriorate following electrical discharge.

The end of nano column can also be used as a spraying tip directly, eliminating any post-column dead volumes [21].

Recently were introduced in the market microfluidic interfaces based on microchip technology. The chip is an integrated system with injection valve, trapping column, analytical column and Nano-ESI tip combined in one relatively small object. In this way 30-50% of the connecting tubes usually used in Nano-LC are eliminated, so minimizing dead volumes [14].

However, the significant cost represents a barrier to their wide-spread adoption [22].

2.5. Recent development in Nano-LC: Portable Nano Liquid Chromatography

The need for analytical instrumentation that allows on-site analysis of various samples, for example for the identification and concentration of pollutants in the environment, has led to the development of portable versions of Nano-LC.

In 1983, Baram et al. introduced the first portable LC. This instrumentation weighed 45 kg and was essentially a vehicle-portable system [23].

Thirteen years later, Baram reported another portable LC, weighed 15kg and was designed for mobile laboratories. The next year, Tulchinsky and St. Angelo introduced "MINICHROM", a portable LC system that weighed 9.5 kg and was 12 V DC battery-operated [23].

Therefore, during past decade several attempts have been made to develop a chromatography that meets the general requirements for portable field instrumentation, i.e. total portability, small size, low power consumption, robustness, reliability and adequate analytical performance [24].

In 2020, was introduced by Lee et al. a commercially available system (Focus, Axcend), equipped with a C18 packed column and UV LEDs for detection at 255 nm and 275 nm.

The innovative instrumentation is very practical due the small size: 20.1 (height) × 23.1 (width) × 32.0 (depth) cm and weighs 7.82 kg.

The fields of application that may benefit from portable Nano-LC are many: pharmaceutical, biological, environmental, forensic.

The work presented in Chapter 6 was carried out by using a hand portable nano-LC for analysis of cannabinoids in inflorescence of Cannabis Sativa L. The objective was to demonstrate the advantages of this technique for analysis in situ, developing a quick and easy sample extraction, using in addition the linear retention indices for univocal identification of target analytes.

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Chapter 3

Superheated water chromatography

3.1. Introduction

In liquid chromatography, an ideal mobile phase should have specific requirements such as:

- non-toxic
- non-flammable
- high purity
- ability to dissolve polar and non-polar analytes
- high speed diffusion
- low cost

Unfortunately, most solvents don't have all of these requirements; in fact, they are often highly flammable, toxic and have a high cost due to their high purity.

To overcome these problems, in 1990, Smith et al. introduced for the first time the use of pure water as the mobile phase at temperatures ranging from 100 to 200°C in reversed-phase liquid chromatography [1].

In the temperature range used for SHWC, dielectric constant values for high-temperature water are similar to those of water–methanol or water–acetonitrile mixtures at room temperature [2].

In addition to the dielectric constant, the solvation parameters of water also change considerably as the temperature changes, allowing the separation of many classes of organic solutes using only water as the mobile phase, without organic modifiers.

The following sections contain a description of theoretical aspects, instrumental components, columns selection and detectors used in SHWC technique.

3.2. Theoretical aspects of SHWC

3.2.1. Water as mobile phase

Pure water as mobile phase is commonly used for both reversed-phase liquid chromatography and normal phase liquid chromatography. In both cases, various organic solvents are mixed with pure water in different proportions to allow the separation of a broad variety of compounds.

The most important novelty introduced by Smith et al. [1] was the elimination of the organic modifier for the separation of non-polar compounds in reversed-phase liquid chromatography.

However, two problems were daunting and needed to be explored: water boiling above 100 ° C and low affinity of this solvent for non-polar compounds. The solution to these problems lies in the physico-chemical characteristics of the water.

As is known, in order to keep water in a liquid state, at temperatures above the boiling point, it is necessary to maintain the pressure above atmospheric values. The operating pressures of the HPLC instrumentation allow to keep the water in a liquid state.

A phase diagram is a graph which shows under what conditions of temperature and pressure distinct phases of matter occur [3]. Phase diagrams are a graphical representation of a physical states of a pure substance or a mixture under different conditions of temperature and pressure and are divided into three single phase regions: liquid, gaseous, and solid states. The lines that separate these single-phase regions are known as phase boundaries. Along the phase boundaries, the matter being evaluated exists simultaneously in equilibrium between the two states that border the phase boundary [3].

For each fluid there is a temperature range, beyond which, regardless of the pressure, it is not possible to maintain the liquid state.

For water, this temperature range is fixed between 0.01 (triple point temperature) and 374 ° C (critical temperature): below this range, even using high pressure, the water will not be liquid and above this range the density of the liquid state and the gaseous state will be very similar. In Figure 3.1 shown the phase diagram of water.

Therefore, high temperature water, at a pressure above atmospheric, has an affinity for non-polar compounds.

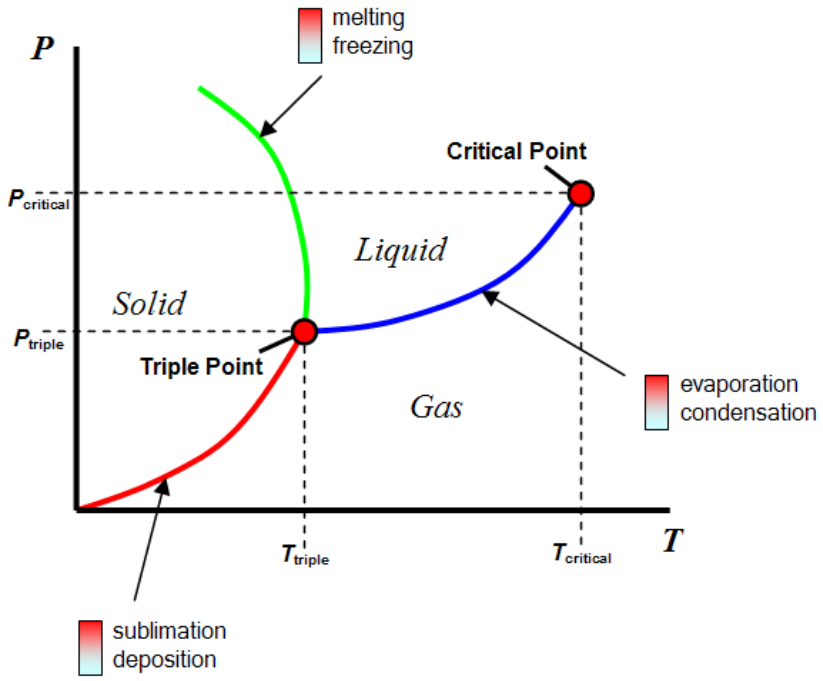

Figure 3.1. *Phase diagram of water*

The polarity of the water (expressed as dielectric constant), increasing the temperature, decreases proportionally; dielectric constant values for high-temperature water are similar to those of water–methanol or water–acetonitrile mixtures at room temperature

A chart of temperature related effects on dielectric constant can be seen in Figure 3.2. At a pressure of 50 bar, the pure water curve is very similar to the water/organic solvent mixture curve: for example, by increasing the temperature from 25 to 125 ° C, the dielectric constant of water decreases from 78 to 49, equal to the dielectric constant of a methanol/water solution, 60:40.

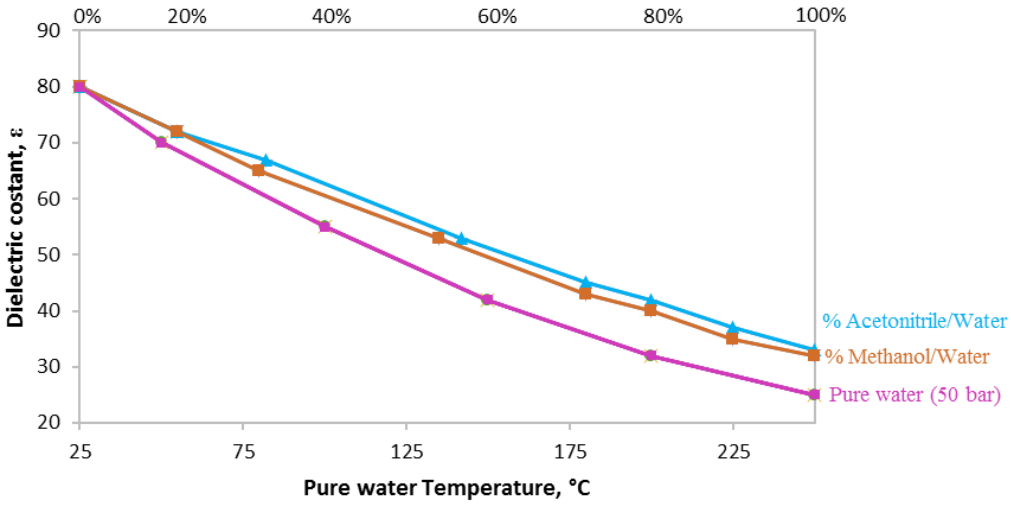

Figure 3.2. *Phase diagram of dielectric costant of water*

In 1994, Hawthorne et al. [4] proposed the water as extraction solvent for non-polar analytes from environmental samples. Supercritical water is very aggressive solvent that readily oxidizes or decomposes many substances. However, at temperatures between 120 and 250 ° C, the target analytes didn't degrade: the recovery values calculated in this temperature range were higher than those calculated at 300 ° C, showing that the intermediate temperatures were better.

The Kamlet-Taft solvent parameters are very useful to study the structural and thermodynamic properties of water. These parameters are π* (or dipolarity/polarizability ratio), α (or hydrogen-bond-donating acidity) and β (hydrogen-bond-accepting basicity). What happens is that the increasing temperature diminishes electrostatic interactions between the water molecules, and also between water molecules and surrounding ions and molecules (i.e. both β and π * decrease and α increase with increasing temperature). An increased movement / rotation of water molecules can also be observed at higher temperature. Hence, the use of liquid water at higher temperature and pressure allows for dissolving less polar compounds, since the intermolecular interactions involving hydrogen bonding becomes less pronounced, thereby favoring London dispersion forces (induced dipole-induced dipole forces) [31]. In other words, liquid water at elevated temperature (and pressure) renders the water a less polar of a solvent [5].

Therefore, the SHWC technique has many advantages: versatility, compatibility with various stationary phases and detectors and low environmental impact.

3.2.2. Influence of temperature on theoretical plate height, retention factor and separation factor

The influence of temperature on the height of a theoretical plates is a crucial point of SHWC technique. In general, in SHWC it is possible to use higher flow rates with a minimum loss of column efficiency, still maintain good resolution [6].

The van Deemter equation has been used to study the effect of temperature on column efficiency.

The dependence of the height equivalent to a theoretical plate (HETP) in dependence on the linear velocity of the mobile phase can be written as:

$$H = A + \frac{B}{u} + Cu \qquad [Eq. 3.1]$$

the A-term again reflects band broadening due to the uniformity of the column packing, the B-term accounts for longitudinal (axial) diffusion, while the C-term represents the resistance to mass transfer in the stagnant mobile phase and stationary phase. A-term does not depend on temperature, B-term is directly proportional to the diffusion coefficient while the C-term is inversely proportional to the diffusion coefficient [7].

The diffusion coefficient is calculated from equation 3.2:

$$D_M \propto \frac{\sqrt{\psi_2 M_2}}{\eta V_1^{0.6}} T \qquad [Eq. 3.2]$$

Where T is the absolute temperature, M_2 is the molecular weight of the solvent, V_1 is the molar volume of the solute in millilitres and η is the viscosity in centipoise. Ψ_2 is the association factor for the solvent, which is 1 for non-polar solvents, 1.9 for methanol and 2.6 for water.

The diffusion coefficient is directly proportional to temperature and also inversely proportional to viscosity, because for a liquid, the viscosity decreases with increasing temperature, thereby also enhancing the diffusion of analytes. The decrease in viscosity of the eluent allows for a high flow rate and these effects result in rapid analyzes without loss of column efficiency.

The influence of temperature on the retention is given by the van't Hoff equation:

$$\ln k = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} + \ln \beta \qquad [Eq. 3.3]$$

where ΔH and ΔS are enthalpy and entropy of transfer of the solute from the mobile into the stationary phase, respectively. R is the ideal gas constant and β is the volume phase ratio of the stationary and mobile phase. Theoretically, a plot of ln k vs. 1/T yields a straight line. In the literature there are examples of linear curves for SHWC [8,9], however the non-linear van't Hoff curve is also very common, in fact many mechanisms govern the retention factor. Changes in pH of the mobile phase and pKa of analytes need to be considered using high temperature, thermal expansion of the column when the temperature is increased, high pressure, the compressibility of the bonded phase has also to be taken into account [7]. In general, for a given solute, the retention factor decreases with increasing temperature. However, it is not possible to define the range of temperature where linearity can be assumed without an experimental proof.

Differences in enthalpies of transfers for different solutes is the key driving force for changes in chromatographic selectivity as a function of temperature, which can be exploited. Deviations from the linear behavior of van't Hoff plots have recently been reviewed and are typically due to different desorption kinetics of different functional groups, dual retention mechanisms, or changes related to the mobile phase that may occur at elevated temperatures [6].

Edge et al. [10] demonstrated that, at a temperature between 40 and 180 ° C using water as mobile phase, the linearity of the elution of the compounds was obtained, for caffeine and aminoantipyrine

an inversion of the order of elution. As they separated at low temperatures, the peaks joined at 113 ° C but as the temperature increased further, they could be separated again [7].

When a curvilinear van't Hoff plot for a compound is obtained, the effect might be even more pronounced [6].

3.3. Instrumentation

3.3.1. Pumps, oven and sample injection

In SHWC technique, water is often used as the only component of the mobile phase and a single pump is required, however, when using a small percentage of organic solvent, two pumps are required as with conventional HPLC instrumentation.

The injection system is the same as conventional HPLC, the sample is normally injected into rotary injection valves mounted externally to the oven at room temperature to prevent boiling or evaporation of compounds.

The most important component of the SHWC instrumentation is the oven. HPLC ovens have a temperature range of up to 80 or 100 ° C. Most SHWC systems have been built around GC ovens, which can usually go up to 350 ° C. The use of these ovens is convenient in isothermal analyzes, but in the temperature gradient it is very difficult to achieve rapid and efficient heating of the column. To overcome these problems, Teutenberg et al. [12] designed an instrument in which a heater was placed in contact with the column, this ensures that the column is heated quickly and easily. An alternative with a closely fitted resistively heated jacket has been used by Harvey-Doyle et al [13].

3.3.2. Eluent preheating

In SHWC it's necessary to efficiently heat the mobile phase before it enters the column to avoid poor reproducibility, cooling effects and other separation problems. In most SHWC either an extended pre-heater coil within the oven or a heater on the inlet line are used to raise the mobile phase temperature. To overcome issues with thermal mismatch, more severe at high eluent flow rates, Thompson et al. suggested that the temperature difference between the incoming eluent and the column should not exceed 5°C [11].

The length of the preheating coil is very important and must be adapted to the flow rate and the internal diameter of the column being used. In 2001, Fields et al. [15] observed that using a short coil of only 15 cm with a volume of 3.4 ul at flow rate of 0.7 ml/min gave distorted peaks, whereas using long coil of 140 cm at flow rate of 1.5 ml/min a good peak shape was obtained.

Years later, Teutenberg [12] used a block heating furnace with the preheating capillary clamped tightly between two aluminum blocks, this is the method most efficient: with only 15 cm of tube is possible heated the mobile phase from 30°C up to 190°C.

More recently, some manufacturers have developed HTLC column heaters that have overcome this problem by using fast response preheaters that require very low volumes and allow preheating of the mobile phase to eliminate thermal mismatch.

3.3.3. Post-column eluent cooling

The final technical challenge is the question of how to reduce the mobile phase temperature post column before it enters the HPLC detector, without causing a significant broadening of peaks or other separation problems. Many studies have proposed home-made solutions for cooling the mobile phase before entering the detector flow cell, for example by dipping the capillary in an ice-bath. However, for routine analysis the temperature has to be controlled more accurately. Technically, this is achieved by using Peltier cooling. Chapter 7 contains a detailed description of the cooling gap used in this research.

3.4. Columns selection

The common stationary phase used in RP-HPLC (i.e. ODS-bonded silica, universally used for analysis at room temperature) are not suitable for analysis at high temperature. The first studies with superheated water employed thermally stable PS-DVB materials. However, these columns showed too retentive character for non-polar compounds. Alternative column materials used for SHWC have included porous graphitized carbon- and zirconia-based phases and hybrid silica type materials.
In this section will be describe the advantages and disadvantages of these stationary phases.

3.4.1. PS-DVB column materials

Most of the early studies used polystyrene divinylbenzene column PLRP-S (PS-DVB) [16-18] filling with crosslinking polymer PRP-1 [19,20]. Thermal stability of these columns is in the range from 100°C to 200°C. However, disadvantage was the high retention capacity, which mean that high temperatures were often needed to achieve elution of moderately polar compound, such as alkyl aryl ketones [2].

3.4.2. Silica-based column materials

Thermal stability of these columns is low. Typical temperature range for these columns is from 50°C up to 150°C. With small addition of an organic solvent this range can be slightly increased to 200°C, however, with 100% water the recommended stability limit is 100°C. More recently, there have been new more stable columns placed on the market, such as Aquatherm Pathfinder C_{18} [21], which where reported to be stable at 200°C, but they appear to have been little used.

3.4.3. Hybrid silica phases

These columns are future phases for SHWC. They have chemical and thermal stability. These supports, which can carry C_{18}, C_8 or phenyl bonded phases which have been successfully used in this technique. The thermal stability is up to 200°C.

3.4.4. Zirconia-based columns

To overcome the thermal stability of silica, other metal oxides have been studied as potential reversed-phase substrates, including aluminia, zirconia and titania. Among them, zirconia-based columns are very interesting. The most commonly used are encapsulated zirconia by polybutadiene (PDB) or bypolystyrene (PS). There are also other zirconia-based materials such as carbon coated zirconia (CARB) or secondary bonded C18zirconia column. Thermal stability of PDB columns has been determined at 200 °C. However, manufacturers recommend using it up to the upper limit of 150 °C.

Kephart et al. [9] showed that capillary PDB and CARB columns arestable up to 370 C and 300 C respectively at the pressure of about 758 bars. They were successfully used for separation of phenol compounds and alkylbenzene.

3.4.5. Carbon-based columns

Porous graphitic carbon (PGC) stationary phases have high thermal stability and they have been successfully used for SHWC. PGC is stable over pH 0–14 and can withstand temperatures >200 °C, thereby enabling use of extreme separation conditions [22]. However, PGC columns have disadvantages such as: contamination problems, peak shape is often asymmetric and the column performance does deteriorate with time, due to difference in thermal expansion of carbon and stainless-steel column material.

3.5. Detectors

One of advantages in using an organic solvent-free eluent is that it makes different detection techniques feasible, such as spectroscopic detectors, FID, ELSD and mass spectrometry.

3.5.1. Spectroscopic detectors

The UV / visible spectroscopic detection is the most used detection method in SHWC. The use of 100% water as the eluent has the advantage that there is no background eluent absorbance [11]. UV detector with a back-pressure regulator was used to prevent the boiling and the rapid evaporation of the mobile phase when it exits the column [23]. Fluorescence detection has been used for vitamins [24] and salicylamide [25].

3.5.2. Flame ionization detectors

One of the principal attractions of the SHWC-FID is the potential to obtain a universal detection for both volatile and involatile analytes. The first studies used the direct capillary transfer of the eluent into the flame, however, to keep the flame burning, it is necessary to increase the hydrogen flow rate (300 mL / min) and control the quantity of water vapour that can be passed to the FID flame without over-loading the combustion. As an alternative, Yang et al. [26] split the effluent from a conventional 2 or 4 mm I.D. column so that only 5–11% of a column flow rate of up to 1.24 mL min^{-1} reached the FID capillary [27].

3.5.3. NMR and MS spectrometric detection

The information available from modern NMR spectrometry makes the combination with HPLC a great method for structural identification of natural products chemistry. Compared to conventional LC, SHWC techniques have more advantages: the possibility of using deuterated water instead of expensive deuterated organic solvents. Because the SHWC system has to be outside the magnetic field, the connecting tube provides sufficient back pressure and cooling so that when the eluent reaches the spectrometer flow cell, it is at ambient pressure and temperature. NMR and MS detections has been used for pharmaceuticals [28], vitamins [29] and nucleic acids [30].

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Chapter 4

Inductively Coupled Plasma-Mass Spectrometry

4.1. Introduction

Inductively coupled plasma – mass spectrometry (ICP-MS) was first developed over 30 years ago, is one of the leading techniques in the field of elemental analysis, focusing on the determination of ultra-trace levels of metals and metalloids in a large variety of sample types.

The most significant advantages of ICP-MS instrumentation, over atomic spectroscopic techniques, such as flame atomic absorption (FAA), electrothermal atomization (ETA) and inductively coupled plasma optical emission (ICP-OES), are the ability to measure multiple elements simultaneously in a single run and determine analyte concentrations down to sub nanogram per liter or parts per trillion (ppt) [1]. Other advantages are: high sensitivity, a wide linear dynamic range and the possibility to obtain isotopic information. However, this technique is also characterized by some disadvantages such as spectral interferences, equipment cost, high level of staff expertise, operating cost. What makes Inductively Coupled Plasma Mass Spectrometry (ICP-MS) unique technique is its ability to sample the analyte continuously, without interruption.

Several models of ICP-MS are available today, which share many similar components, such as nebulizer, spray chamber, plasma torch, and detector, but can differ quite significantly in the design of the interface, ion focusing system, mass separation device, and vacuum chamber [2]. Figure 4.1 shows the detection limits of all the elements that can be detected, together with their isotopic abundance. The principles of operation and instrumental components will be discussed in the next section.

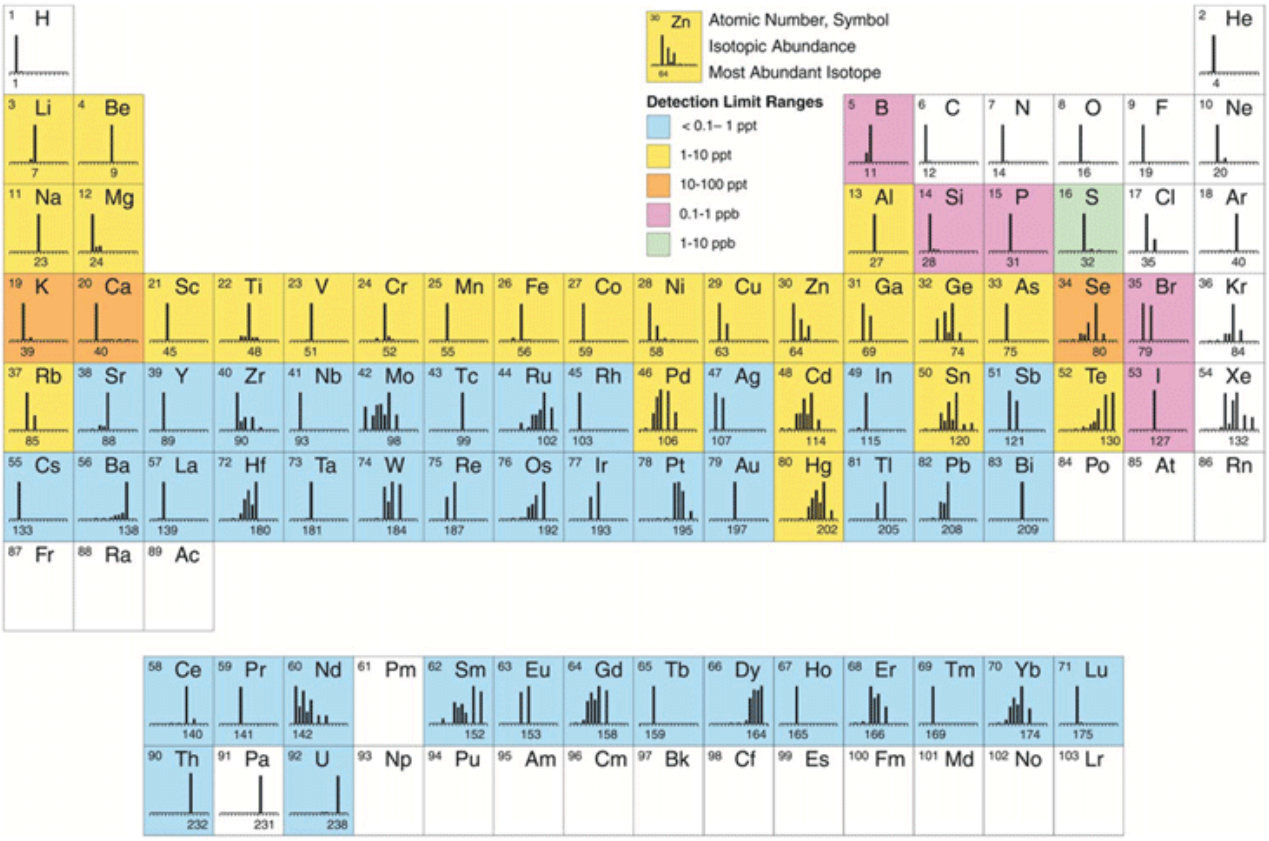

Figure 4.1. *Approximate detection capability of ICP-MS, together with elemental isotopic abundances*

4.2. Sample preparation

Sample preparation for ICP-MS consists of the dilution or thermal digestion of biological samples prior to analysis. The extraction procedure of metals from organic samples and elements' determination by ICP-MS is difficult, and one of the most challenging analytical problems. Most matrix is characterized by: a high organic load that increases the matrix effects and the possibility of polyatomic molecular interferences from elements like C, N and S. High organic content can result in carbon deposition on the sampling cone and the loss of sensitivity.

Deionized water has been used as a diluent, however some elements are unstable in pure water, therefore acidic or alkaline diluents are preferred in most cases [3]. More common is acid digestion with a microwave system. By using concentrated acids or mixtures thereof, the matrices of organic and inorganic samples can be totally destroyed or dissolved, and the whole sample can be brought into solution.

The most commonly used acids for acid digestion and their typical concentrations are:

- Nitric acid, HNO_3 (65 %)
- Sulfuric acid, H_2SO_4 (95 % to 98 %)
- Perchloric acid, $HClO_4$ (70 % to 72 %)
- Hydrochloric acid, HCl (30 % to 37 %)
- Hydrofluoric acid, HF (40 % to 48 %)
- Phosphoric acid, H_3PO_4 (85 %)
- Boric acid, H_3BO_3 (approx. 5 %)
- Hydrogen peroxide, H_2O_2 (30 %)
- Aqua regia, HCl + HNO_3 (volume ratio 3:1)
- Reverse aqua regia, HCl + HNO_3 (volume ratio 1:3)

Microwave digestion mineralizes a wide variety of samples including food, clinical / biological sciences, environment, geosciences and mining, metallurgy, pharmaceuticals / nutraceuticals, paints and coatings, plastics and polymers. However, the disadvantages are the need for a high level of staff competence, the cost of acid solvents and the high environmental impact.

4.3. Sample introduction

The first step in ICP-MS is sample introduction. ICP-MS instrumentation today is primarily designed for analyzing liquid samples. The analysis of solid samples is performed by laser ablation or electrothermal evaporation. Before being introduced into the ICP-MS, the solutions are converted into a fine aerosol, so that they can be efficiently ionized in the plasma discharge. The sample is pumped with a peristaltic pumps at 1 mL/min into a nebulizer, where it is converted into a fine aerosol with argon gas at approximately 1 L/min. The fine droplets of the aerosol are separated from larger droplets by means of a spray chamber and only 1-2% of the sample reaches the plasma. This section contains a more detailed description of the nebulizer and spray chamber.

4.3.1. Nebulizers

The liquid sample is converted into a fine aerosol by the nebulizer. There are several commercially available nebulizers, including pneumatic, ultrasonic nebulizers, and desolvating system.

Each nebulizer has advantages and disadvantages related to application.

Pneumatic nebulizers are the most common and used. Some of the most popular designs of pneumatic nebulizer include the concentric, microconcentric, microflow, and crossflow. They use the force of a flowing gas (normally argon at a pressure of 20–30 psi), passing through an orifice or capillary tube, to create microdroplets from the liquid sample. These droplets are transported via the flowing gas stream to the plasma for decomposition, atomization, and ionization [4]. The advantages of pneumatic nebulizers are excellent sensitivity, stability and rugged for routine use. The disadvantage is they can be plagued by blockage problems, especially if large numbers of heavy matrix samples are being aspirated.

Ultrasonic nebulizers use the sound energy of a piezoelectric transducer to generate the aerosol. These nebulizers improve analytical sensitivity by an order of magnitude over pneumatic nebulizers, however they are significantly more expensive [1].

Desolvating nebulizers use a heated spray chamber to desolvate the sample before it reaches the plasma [5]. The desolvating nebulization systems allow to use low sample flow rates and have better detection limits than the pneumatic ones; however, the accuracy is lower, so the detection limits are not good. Volatile analytes can be lost due to high heater temperatures.

4.3.2. Spray chambers

The plasma is inefficient at dissociating large droplets: the spray chamber performs this function and acts to smooth out nebulization ‘pulses’ produced by the peristaltic pump [1]. Today, commercially available spray chambers are the Double Pass and Cyclonic systems.

In a double pass spray chamber, the small droplets from the nebulizer are directed into a central tube. The large droplets exit the spray chambers by gravity and an external drain remove condensed solvent. The cyclonic spray chamber works similar, but operating by centrifugal force.
Smaller droplets are carried with the gas stream into the ICP-MS, while the larger droplets impinge on the walls and fall out through the drain [2].
The temperature of the spray chamber is very important because it minimizes the formation of oxides and avoids overloading the plasma with solvent. The temperature of the spray chamber is normally maintained around 2 ° C [1].

4.4. The plasma torch and ionization

The plasma torch is the most important part of the ICP-MS instrumentation. The role of the plasma in ICP-MS is to ionize the sample. The plasma torch consists of three concentric tubes, which are normally made from quartz. A typical quartz torch is shown in Figure 4.2.

The argon gas flowing into the space between the outer and center tubes for isolated the plasma from the internal wall of the outer quartz tube preventing melting, and it encourages the formation of an annular-shaped plasma. The auxiliary gas passes between the middle tube and the sample injector and is used to change the position of the base of the plasma relative to the tube and the injector [2]. The center tube is for the injection of sample aerosol into the plasma [5].

The plasma torch is mounted horizontally and positioned centrally in the RF coil, approximately 10–20 mm from the interface [2].

The ICP is considered a “hard” ionization technique because utilizes a high-temperature plasma that leads to a complete fragmentation of sample molecule, leaving only the detectable, atomic constituents, namely metals, metalloids or heteroatoms.

Radio frequency (RF) applied to the copper coil produces an intense electromagnetic field. An interaction between this electromagnetic field on a spiral (tangential) flow of argon gas directed between the outer and middle tube of a quartz torch is how the plasma is formed.

A high voltage spark applied to the argon is necessary for the ionization of the gas. Argon flows throughout the quartz torch; electrons and cations produced accelerate towards the RF coil. The cations and electrons collide with other argon molecules in a chain reaction during this acceleration, forming what is known as an ICP discharge. With ample argon supplied, the plasma will reach equilibrium and remain at a constant temperature of about 6.000°C for all the analyses time.

With regard to the sample, the fine aerosol is swept in a stream of argon gas along the injector and into the plasma. After reaching the high-temperature plasma, the sample is desolvated, vaporised, atomised and ionized [1]. The degree to which an element is ionised depends on the temperature of the plasma and the ionisation potential of the element [1].

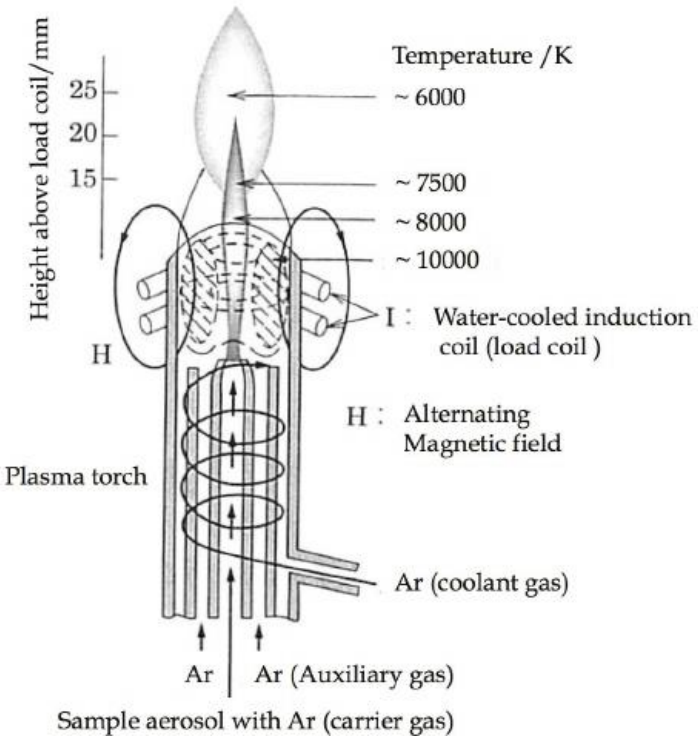

Figure 4.2. *Detailed view of plasma torch*

4.5. Interface

The interface region is the point at which the ions efficiently, consistently and with electrical integrity to transport from the plasma, which is at atmospheric pressure (760 Torr) to the mass spectrometer analyzer region at approximately 10^{-6} Torr.

The interface consists of a pair of coaxial nickel (or platinum) cones that separate the plasma from the mass spectrometer vacuum chamber. The first cone is called sample cone, and the second is called the skimmer cone. The sample is transported from the plasma into the interface region through a small orifice into the first cone (0.8-1.2 mm i.d.). From there, the sample is transported into a skimmer cone (0.4-0.8 mm i.d.). The ions emerge from the skimmer cone and can be guided effectively by charged surfaces called electrostatic lenses into the mass separation device. The interface region is shown in Figure 4.3.

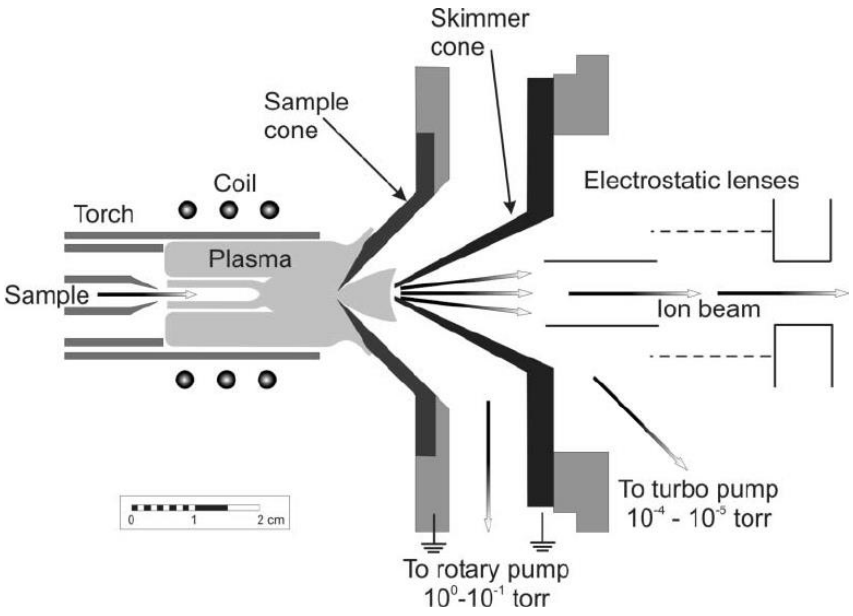

Figure 4.3. *Detailed view of interface region.*

4.6. Ion optics

The ion beam is electrostatically focus toward the MS device by the ion optics.

The ion optics are positioned between the skimmer cone and mass analyzer and maintained at a vacuum of approximately 10^{-3} Torr with a turbomolecular pump.

The ion optics made up of a series of metallic plates, barrels or cylinders, which have a voltage placed on them. The function of the ion optic system is to take ions from the plasma at atmospheric pressure via the skimmer cone and steer them into the mass analyzer, which is under high vacuum

The ion optics prevents that the particulates, neutral species, and photons reach the detector either by using some kind of physical barrier, by positioning the mass analyzer off axis relative to the ion beam or by electrostatically bending the ions by 90°C into the mass analyzer. These species, in fact, if reach the detector cause signal instability elevate the noise of the background and therefore degrade detection capability [2].

4.7. Mass analyzer: quadrupole mass filter technology

The mass analyzer is positioned between the ion optics and the detector and is maintained at a vacuum of approximately 10^{-6} Torr with an additional turbomolecular pump to the one that is used for the lens chamber. Today, the most common mass separation devices used in ICP-MS analysis is the quadrupole mass filter.

The principle of the quadrupole mass analyzer was described for the first time by Paul and Steinwegen in 1953 [6].

The mass analyzer consists of four parallel hyperbolic or cylindrical metallic rods of the same length and diameter and arranged in a square array (Figure 4.4.); They are typically made of stainless steel or molybdenum; quadrupoles used in ICP-MS are typically 15–20 cm in length, about 1 cm in diameter, and operate at a frequency of 2–3 MHz. Each pair of opposing rods is held at the same potential which is composed of a direct current (DC) and a time dependent alternating current (AC) component. By selecting the optimal AC / DC ratio on each pair of rods, ions with a particular m / z ratio pass through the rods to the detector, while the others are unstable, collide with the rods and are ejected from the quadrupole.

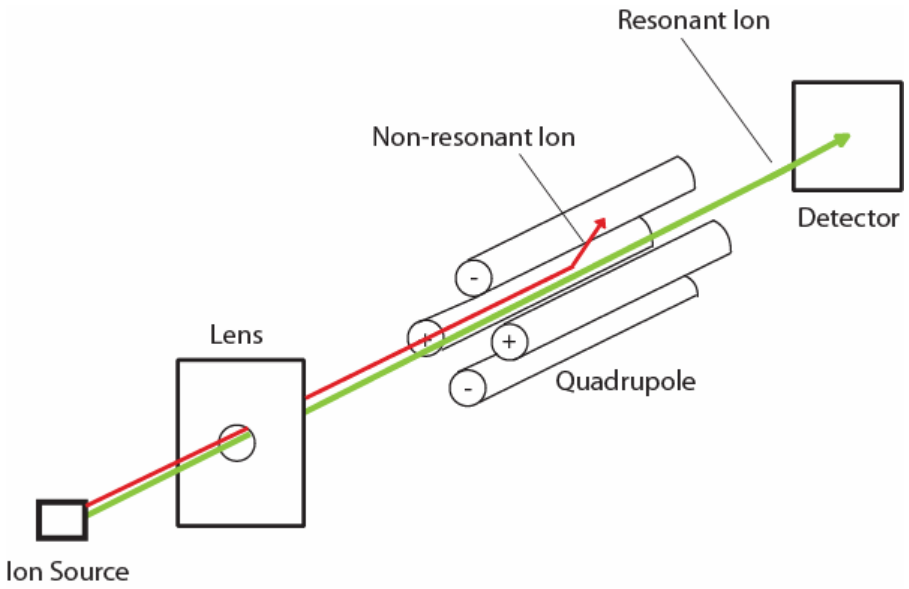

Figure 4.4. *Schematic representation of a quadrupole mass analyzer.*

There are two important performance specification of a mass analyzer. The first is the resolution, which is for the quadrupole analyzers is the width of a peak at 10% of its height and is normally approximately 0.75 amu (atomic mass units). The second is abundance sensitivy, which is the contribution that a signal for an isotope at a certain mass (M) makes to adjacent masses (*M*-1, and *M*+1) [1]. For quadrupole analyzer is typically 1×10^{-6} at *M*-1 and 1×10^{-7} at *M*+1. In other words, an interfering peak of 1 million counts per second (cps) at *M*-1 would produce a background of 1 cps at

M, while it would take an interference of 10 million cps at M+1 to produce a background of 1 cps at *M* [2].

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Chapter 5

Non-psychoactive cannabinoids identification by linear retention index approach to a hand-portable capillary liquid chromatography platform†

5.1. Introduction

In the past decade, the role of cannabinoids in the human body has received a lot of attention. The scientific community has focused on the potential benefits and risks [1].

Cannabinoids are natural compounds that belong to the biochemical class of meroterpenoids [2]. They are the most important secondary metabolites of the *Cannabis sativa* L. plant species [3].

The taxonomic classification is more difficult; was previously classified as Urticaceae, then Moraceae, and was only recently classified as Cannabaceae [4]. The chemotypes of C. Sativa L. differ based on the cannabinoid profile. This classification takes into account the concentration of Δ^9-tetrahydrocannabinol (Δ^9-THC), the principal psychoactive cannabinoid of cannabis. [2,5]. In particular, there are two types of Cannabis: one known as industrial hemp, with a Δ^9-THC content equal to or lower than the legal limits of 0.2/0.3%, used for textile or food purposes, and one, known as drug-type Cannabis, used for medicinal or recreational purposes, with a high concentration of Δ^9-THC [6]. New cannabis varieties are continuously formed due to the repeated interbreeding of these species, variations in climatic conditions, and the wide spread of this crop, and, for this reason, their phytochemical composition must be studied for both legislative and therapeutic purposes.

The inflorescences of *Cannabis Sativa* L. contain high concentrations of cannabidiolic acid (CBDA) and its decarboxylated forms of cannabidiol (CBD). CBDA is the main non-psychoactive constituent in the fiber-type *Cannabis* [5]. The effects of CBDA on the body are still being researched. Several studies have shown that they inhibit the cyclooxygenase-2 (COX-2) enzyme. These enzymes are associated with inflammation, the inhibition can relieve pain [7]. While the anti-inflammatory, antibacterial, and anxiolytic effects of CBD are well known [8].

Although at low concentrations, cannabigerolic acid (CBGA) and its decarboxylated derivative, cannabigerol (CBG) have been found in this plant [3,8,9]. The role of the CBGA has not yet been clarified. A study showed that this cannabinoid could play an important role in the inhibition of aldehyde reductase, which has been under study for several years in the complications of diabetes. [10] Furthermore, it has been found to have cytotoxic effects [11]. CBG appears to promote CBD-like effects as an anxiolytic, antineoplastic and pain reliever [12].

In this work, minor cannabinoids were also studied and identified, including cannabichromene (CBC), having anti-inflammatory effects [13], cannabinol (CBN), mildly psychoactive, soothing for inflamed skin, antibacterial and anticonvulsant [13], tetrahydrocannabinolic acid (THCAA), with properties neuroprotective [14]. Figure 5.1 shows the chemical structure of all the cannabinoids under investigation in this study.

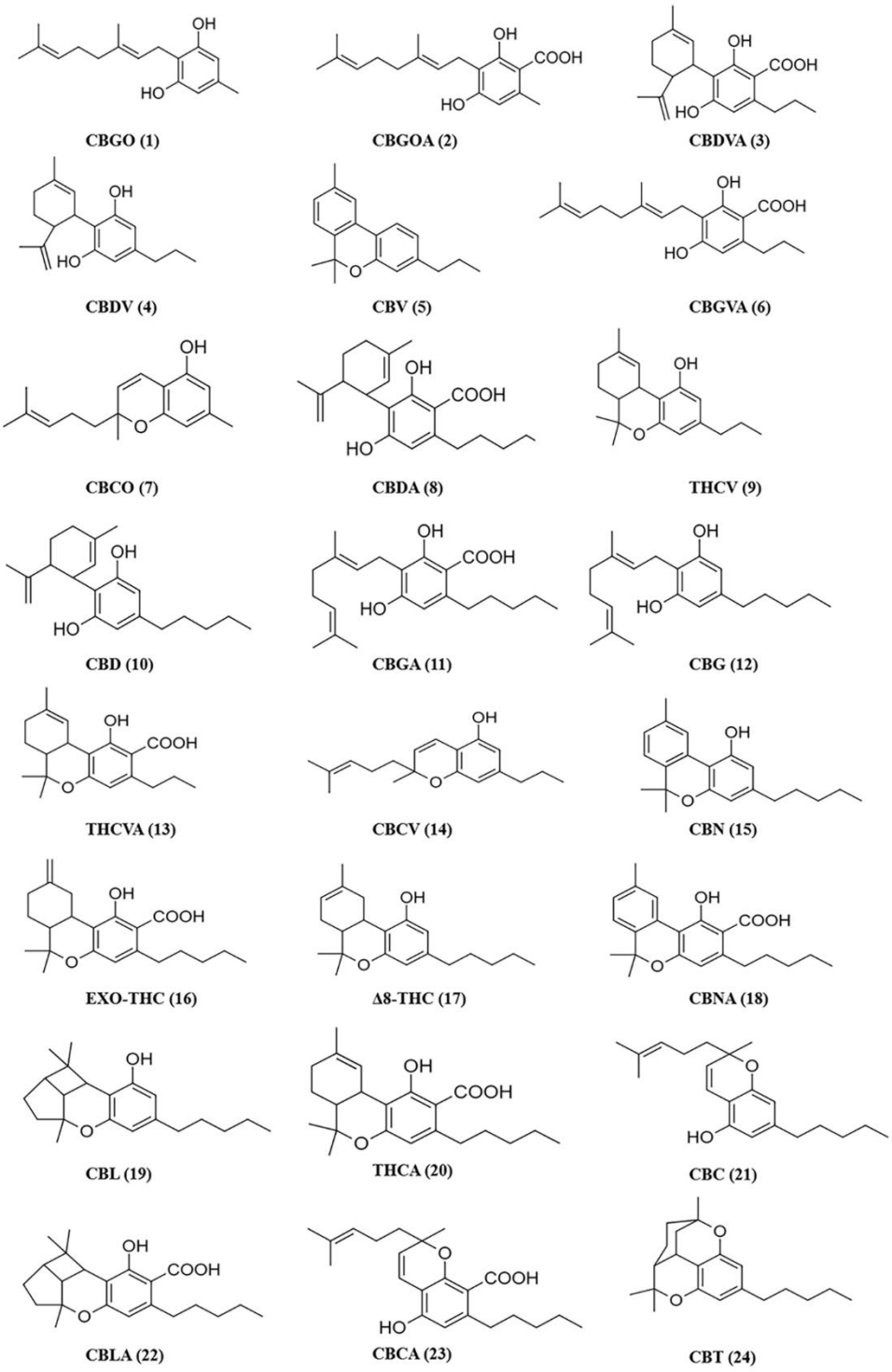

Figure 5.1. *Structures of non-psychoactive cannabinoids included in the present study.*

In recent years, due to the increased interest in this plant, used for both recreational and therapeutic purposes, it has become necessary to develop and validate fast and reliable methods for phytochemical characterization.

Gas chromatography (GC) methods are commonly used for the analysis of cannabinoids [9,15], but these methods have some limitations: 1) for polar and non-volatile compounds, a derivatization step

is required, resulting in an overall increase in analysis times. 2) It is not possible to determine the acid forms; in fact, they undergo a rapid decarboxylation and conversion to neutral forms due to the high temperatures used [9].

There are several liquid chromatographic methods that have been optimized with some success in terms of efficiency, speed of analysis, and determination of the total composition of cannabinoids [2,16].

The goal of this study was to develop a fast and reliable nano-LC method that combines the advantages of liquid chromatography with the advantages of miniaturization. The advantages of this technique are many: 1) small sample quantities are enough, 2) mobile phase consumption is reduced, resulting in less waste production, 3) short analysis times [17].

In addition, a hand-portable instrumental setup also allows for *in-situ* analyses, and in this work, a method has been utilized that requires quick and easy sample extraction [5]. The performance of this system was therefore evaluated with UV detection at 255 and 275 nm. In addition, linear retention index (LRI) was used for univocal identification of target analytes. The linear retention system was proposed by Kovàts in 1958 [18]. This approach, already widely used in GC, provides for the univocal identification of the target analytes through the retention time referred to the two compounds of the homologous series that elute immediately before and immediately after [19]. In GC only the stationary phase influences the retention time of the analytes, and the combination of the mass data, generally obtained from the electron ionization source, determines a unique identification. This strategy is relatively new in LC and it gets more impetus from the recent progress in LC in the packing of the columns and in analytical instrumentation, which considerably increases the reproducibility of the retention parameters. The task of researcher is to choose the homologous reference series most suitable for the application; this must cover the entire chromatographic space and have a retentive behavior similar to the target analytes [20].

In the present research, the LRI approach was applied for the first time to a hand-portable miniaturized system for identification of cannabinoids in Cannabis sativa L. inflorescences. In particular, two different setups were explored: 1) a single column setup which exploited the sub-2 μm packaging to increase the chromatographic resolution; 2) a dual-column setup based on the serial connection of two different stationary phases, each coupled to a UV detector, to increase the identification power since compounds with the same LRI on the first column could be discriminated on the second one. Moreover, since two different wavelengths were set on the UV detectors, the ratio of the absorbances measured on each chromatographic trace represented a third identification criterion, thus fulfilling the recommendations of the Scientific Working Group for The Analysis of Seized Drugs (SWDRUG)

about the categories of analytical techniques to be used and the minimum number of parameters required for the unambiguous identification of drugs [21].

5.2. Materials and Methods

5.2.1. Chemicals

Standards of cannabidiolic acid (CBDA), Δ^9–tetrahydrocannabinolic acid (Δ^9–THCA), Δ^8–tetrahydrocannabinol (Δ^8-THC), tetrahydrocannabivarin (THCV), cannabidivarin (CBDV), cannabidivarinic acid (CBDVA), cannabidiol (CBD), cannabigerol (CBG), cannabigerolic acid (CBGA), cannabinol (CBN), cannabichromene (CBC), cannabichromenic acid (CBCA), cannabicyclol (CBL), cannabinolic acid (CBNA), tetrahydrocannabivarinic acid (THCVA), cannabicyclolic acid (CBLA) and exo-tetrahydrocannabinol (Exo-THC), acetophenone (C8), propiophenone (C9), butyrophenone (C10), valerophenone (C11), hexanophenone (C12), heptanophenone (C13), octanophenone (C14), nonanophenone (C15), decanophenone (C16), undecanophenone (C17) and dodecanophenone (C18) were obtained from Merck Life Science (Merck KGaA, Darmstadt, Germany). Cannabigerorcinic Acid (CBGOA), cannabigerovarinic acid (CBGVA), cannabichromevarin (CBCV), cannabivarin (CBV), cannabichromeorcin (CBCO), cannabigerorgin (CBGO) and cannabicitran (CBT) were purchased by LGC standards (Molsheim, France). All standards were certified with the Cerilliant® trademark and were provided as 1 mg/mL solution in methanol or acetonitrile. Trifluoroacetic acid (TFA), water, and acetonitrile were LC-MS grade and were also provided by Merck Life Science (Merck KGaA, Darmstadt, Germany).

5.2.2. Sample preparation

Hemp inflorescences of Tisza variety were purchased from an online smart shop and hemp inflorescence of Finola variety was provided by Canapar (Ragusa, Sicily, Italy). Cannabinoids were extracted from hemp inflorescences (0.5 g), preliminarily ground, with 10 mL of ethanol at room temperature for 15 minutes. The residue was extracted two more times with 10 and 5 ml of ethanol, respectively; then, the three extracts were pooled, dried by using a rotary evaporator (Hei-VAP Precision, Heidolph) and reconstituted in 500 µL of acetonitrile. Standard mixture solutions were prepared by adding 100 µL of each cannabinoid standard solution into a 1 mL volumetric flask, to obtain a final concentration of 100 µg/mL for each compound. All solutions were stored at +4°C until use. Stock solutions of each alkyl aryl ketone were prepared at a concentration of 10 mg/mL; then, a standard mixture (*viz.* the homologous series of alkyl aryl ketones) was prepared to be injected in the single-column cap-LC setup, by mixing different volumes of each stock solution to a final concentration of 1 mg/mL for acetophenone, propiophenone, butyrophenone, valerophenone, decanophenone and undecanophenone, 0.5 mg/mL for hexanophenone, heptanophenone, octanophenone and nonanophenone and 2 mg/mL for dodecanophenone, in order to generate a similar signal for all the compounds. Another homologue series solution was prepared to be injected into the

dual column cap-LC setup, by mixing the standards to a final concentration of 0.5 mg/mL for butyrophenone, valerophenone, hexanophenone and heptanophenone, 1 mg/mL for octanophenone and nonanophenone, and 2 mg/mL for decanophenone, undecanophenone and dodecanophenone.

5.2.3. Instrumentation and analytical conditions

The analyses were carried out on an Axcend Focus LCTM LC system (Axcend Corp, Provo, UT, USA). The dimensions of such hand-portable instrumentation are as follows: 20.1 (height) x 23.1 (width) x 32.0 (depth) cm and 7.82 kg (weight). The LC configuration is equipped with two high-pressure syringe pumps (maximum operating pressure of 6000 psi (410 bar)), connected to a mixing valve (which mixes the two mobile phases contained in reservoirs located in the front part of the instrument) and an injection port with internal sample loop of 40 nL. The sample is manually injected via a 25 µL Hamilton syringe into the injection port. The system is equipped with a cartridge in which both the fused silica capillary column (or the columns) and the on-capillary UV detector(s) are placed. The cartridge can be easily inserted and removed from the rear part of the instrument and correctly positioned thanks to a knob located on the front. In this way, it is possible to fast switch from the single-column to the double-column cartridge. The single column cartridge contains a C18 column (10 cm×150 µm, 1.7 µm dp) and a UV detector set at 255 nm, while the dual column cartridge consisted of a C8 column (10 cm×150 µm, 3 µm dp), followed by an on-capillary UV detector set at 255 nm, followed by a biphenyl column (5 cm×150 µm, 3 µm dp) and an on-capillary UV absorbance was measured at 275 nm. The pictures of both cartridges are shown in Figure S1 (Supporting information). Data were acquired and processed by Axcend Focus V 2.0.3 software. The analyses were performed in a gradient elution on both cartridges, by using as mobile phase the mixture of solvent A (H_2O/ACN 97:3 v/v) and solvent B (ACN/H_2O 97:3 v/v), both acidified with 0.1% TFA. The following gradient was used in the single-column setup: 0-35 min 40%-97% B, 36 min, 40% B. The analyses on the dual column setup were carried out under the following gradient elution program: 0-35 min, 5%-95% B, 36 min, 95% B. The flow rate was 2 µL/min and the initial pressures of the system were 2000 psi (138 bar) and 4000 psi (276 bar) in the single and dual column setup, respectively.

5.2.4. Linear retention index calculation and database building

Linear retention indices were calculated according to eq. 5.1 [29], by using the alkyl aryl ketones homologue series and by assigning to *z* a value equal to the carbon number of alkyl aryl ketone that elutes immediately before the analyte. Then, t_{Ri} is the retention time of the analyte, t_{Rz} is the retention

time of the alkyl aryl ketone eluted immediately before the analyte, and $t_{R(z+1)}$ is the retention time of the alkyl aryl ketone that elutes after the analyte.

$$LRI = 100(z + \frac{t_R - t_{Rz}}{t_{R(z+1)} - t_{Rz}}) \qquad Eq.5.1$$

The LRIs were calculated by injecting the homologous series before and after the cannabinoid standard mixtures under the same analytical conditions.

5.3. Results and discussion

5.3.1. Capillary LC analysis of cannabinoids and building of a linear retention index database on the single-column setup

The LRI identification approach has been widely used in GC for the identification of volatile compounds since it was introduced by Kovatz in 1958 [18]. Contrarily, various attempts to apply RI in LC have been described in the literature, but any of them have received wide acceptance from the scientific community. This was mainly due to the lower chromatographic efficiency of LC packed columns compared to GC capillary columns, but also to the poorer repeatability of LC retention times, which was strongly dependent on the mobile phase composition [20].

The use of LRI has been debated for more than 50 years. Only recently has LRI been considered for identification purposes after an assessment of its repeatability both intra and interlaboratory. [22-25]. For LRI calculation, homologue compounds with chemical-physical features similar to the analytes are used. In this regard, most of the literature in this field employs alkyl aryl ketones for the analysis of small molecules [25]. Cannabinoid analysis is a big challenge due to their very similar chemical structure, which makes it difficult to determine their baseline separation.

A miniaturized instrument setup was used in this study to improve peak resolution and chromatographic efficiency. In this regard, two different configurations were investigated. The first one consisted to the use of a sub-2 μm column to maximize the chromatographic efficiency, while the second one makes use of two serially connected columns, packed with two different stationary phases and two different on-column UV detectors, to increase selectivity.

Starting from the single column setup, Figure 5.2 shows the chromatographic profile of the alkyl aryl ketone reference standard mixture (from valero- (C11) to dodecanophenone (C18) to cover the entire chromatographic space) (Figure 5.2A) along with the profile of the 24 cannabinoid standards (Figure 5.2B).

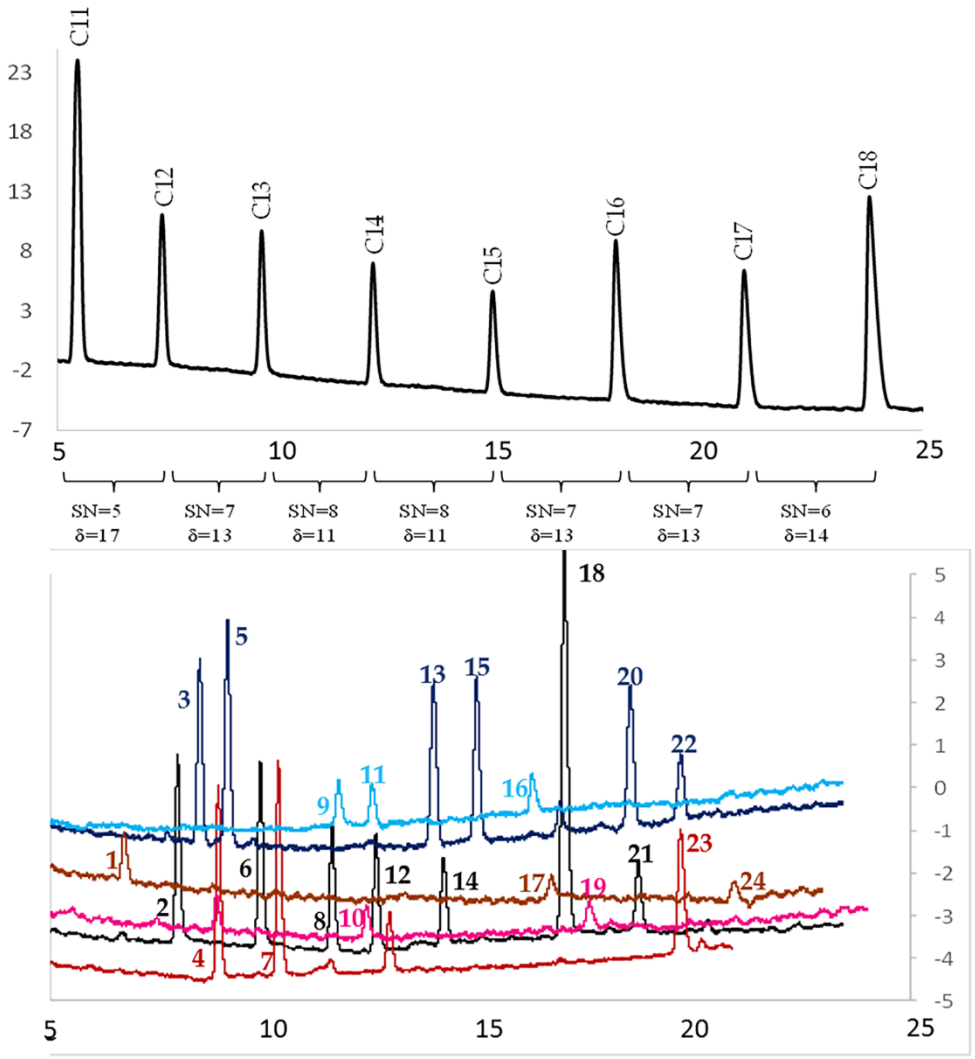

Figure 5.2. *(A) Chromatographic profile of the alkyl aryl ketone homologue series, along with SN and δ values and (B) chromatograms of cannabinoid standard mixtures, on the single column setup. For peak numbering, see Table 6.1.*

Most of the compounds were satisfactorily separated in about 20 minutes. Few exceptions regard compounds 11-12, 13-14, 20-21 and 22-23, *viz.* the critical pairs CBGA and GBG, tetrahydrocannabivarinic acid (THCVA) and cannabichromevarin (CBCV), tetrahydrocannabinolic acid (Δ^9-THCA) and cannabichromene (CBC), and cannabicyclolic acid (CBLA) and cannabichromenic acid (CBCA). In fact, according to a recent review, LC-UV methods have been used to analyze up to 19 natural cannabinoids simultaneously [26]. CBCA and THCVA were only considered in the most comprehensive studies, CBLA was only considered in three case studies, and CBCV was only considered in one of them [27-29]. Critical pairs CBGA/CBG, and Δ^9-THCA/CBC showed near baseline separation even though a mathematical estimate of resolution was not provided. Possible mismatches in cannabinoid identification can occur due to similar retention behaviors of these compounds, as reported in previous research work [30-35].

Table 5.1 shows the LRI values determined for each cannabinoid, as well as their standard deviations (based on four replicates at two concentration levels), which are all less than five units. For 17 compounds, the difference between adjacent peaks was higher than 12 LRI units, between 9 and 12 for 3 cannabinoids, and less than 6 units for the previously mentioned crucial pairs. In order to establish if such a difference allows for the unambiguous identification of unknowns in real-world samples, the minimum retention index difference between baselines resolved adjacent peaks was calculated, basically related to the chromatographic resolution of the developed method. Actually, when the LRI strategy is adopted, the use of the separation number (SN) parameter can be more useful to provide information about the number of compounds that can be satisfactorily separated in a time region and the difference, in terms of retention index, between adjacent resolved peaks.
The following equation is used to calculate SN (Eq. 5.2):

$$SN = \frac{t_{R(z+n)} - t_{Rz}}{w_{h(z+n)} + w_{hz}} - 1 \qquad Eq. 5.2$$

where $t_{R(z+n)} - t_{Rz}$ is the retention time difference between a homologue pair of the reference series, and w_{hz} and $w_{h(z+n)}$ are the widths at half height of the homologue peaks. The minimum retention index difference (δ) between adjacent resolved peaks will be equal to the retention index difference (ΔLRI) in the considered elution region, divided by SN+1. As shown in Figure 2 A, being 100 the ΔLRI between each homologue pair, δ is 100/(SN+1) and ranges from 11 to 17. Such calculations proved that the developed method is able to reliably identify the majority of the compounds under investigation since their LRI difference is higher than δ. Moreover, the minimal LRI variability (≤ 5) also avoids identification errors since it is largely smaller than δ, so that any overlapping between LRI intervals can occur. As a consequence, an LRI tolerance of 5 units will be considered a proper criterion for the automatic identification of cannabinoids in real matrices (section 5.3.3).
The method developed in this research allows the analysis and identification of target compounds, using a miniaturized LC instrumental setup. Compared to conventional techniques, which operate at high flows (from 0.4 ml/min to 1.5 ml/min), only 40 μL of solvent are required in this research.
The longer analysis time (25 min) is justified by the use of the LRI approach, which requires a satisfactory separation in terms of LRI units and entails the use of a linear gradient able to provide uniform coverage of the entire chromatographic space, where uniform means that the homologue peaks should be quite equidistant. This guarantees a similar SN between each homologue pair, so that δ will be almost the same in different time regions (Figure 5.2).

Table 5.1. *LRI values of cannabinoids on the single-column setup.*

Peak number	Compound name	Abbreviation	LRI (n=4)
1	Cannabigerorcin	CBGO	1190±4
2	Cannabigerorginic Acid	CBGOA	1221±1
3	Cannabidivarinic Acid	CBDVA	1248±2
4	Cannabidivarin	CBDV	1261±5
5	Cannabivarin	CBV	1276±2
6	Cannabigerovarinic Acid	CBGVA	1304±2
7	Cannabichromeorcin	CBCO	1324±1
8	Cannabidiolic Acid	CBDA	1369±2
9	Tetrahydrocannabivarin	THCV	1380±2
10	Cannabidiol	CBD	1401±4
11	Cannabigerolic acid	CBGA	1407±2
12	Cannabigerol	CBG	1408±1
13	Tetrahydrocannabivarinic Acid	THCVA	1460±2
14	Cannabichromevarin	CBCV	1465±2
15	Cannabinol	CBN	1497±2
16	EXO- Tetrahydrocannabinol	EXO-THC	1543±3
17	Δ^8-Tetrahydrocannabinol	Δ^8-THC	1555±3
18	Cannabinolic Acid	CBNA	1564±2
19	Cannabicyclol	CBL	1588±3
20	Tetrahydrocannabinolic Acid	Δ^9-THCA	1626±1
21	Cannabichromene	CBC	1625±3
22	Cannabicyclolic Acid	CBLA	1668±1
23	Cannabichromenic Acid	CBCA	1664±5
24	Cannabicitran	CBT	1702±2

5.3.2. Application on a real sample

The ethanol extracts of two different varieties of hemp inflorescences were injected into the single-column setup to evaluate the developed method.

Eight and four cannabinoids were reliably identified in the analyzed samples, in accordance with typical profiles of CBD-chemotypes [32,35,36], also known as fiber-type Cannabis [6].

Figure 5.3 shows the peaks identified in the Tsiza variety sample using the new ChromLinear software. The parameters for the calculation of the LRI are set directly in the new software: the previously integrated chromatogram of the alkyl aryl ketone homologue series has to be loaded and a table with the retention time of the reference compounds is automatically filled with the z and LRI values (equal to z×100) assigned to each reference compound; in this way, the software is able to apply eq. 1 to calculate the LRI of all the unknown peaks; in the same window the LRI library must be loaded for the match between calculated and tabulated LRI. The LRI tolerance for the automatic identification was set equal to ± 5 units, according to the obtained LRI standard deviations (Table 5.2).

Table 5.2. *Identified compounds on fresh inflorescences (sample 1 refers to Tsiza variety and sample 2 refers to Finola variety)*

Peak	Compound name	$LRI_{Sample\ 1}$	$LRI_{Sample\ 2}$	LRI_{Tab}	$\Delta LRI_{Sample\ 1}$	$\Delta LRI_{Sample\ 2}$
1	CBGOA	1216	-	1221	-5	-
6	CBGVA	1303	-	1304	-1	-
7	CBCO	1319	-	1324	-5	-
8	CBDA	1365	1368	1369	-4	-1
10	CBD	1400	-	1401	4	-
15	CBN	1498	-	1497	1	-
18	CBNA	1566	1564	1564	2	0
20	Δ^9-THCA	1625	1622	1626	-1	-4
23	CBCA	-	1663	1664	-	-1

In detail, CBGOA, CBGVA, CBCO, CBDA, CBD, CBN and CBNA were the only candidates for the calculated LRI. On the other hand, two candidates were obtained for peaks at LRI 1625/1622 (sample 1/sample 2) and 1663 (sample 2), corresponding to the above discussed critical pairs Δ^9-THCA/CBC and CBCA/CBLA, respectively.

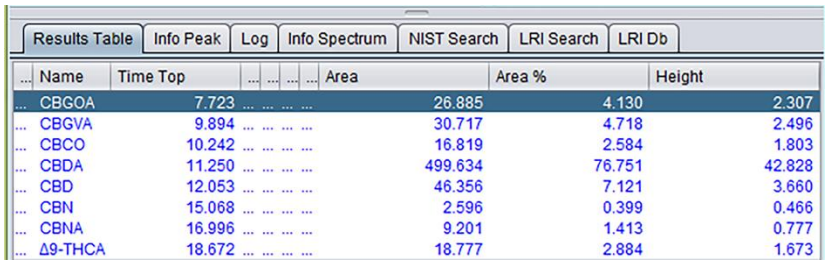

Figure 5.3. *Screenshot of the ChromLinear software, reporting the results table containing the identification of the integrated peaks.*

5.3.3. Preliminary results on the dual column setup

A dual column setup was tested to improve the identification power of this system and eliminate coelution issues. Dual column/dual detection system was used to provide two distinct chromatograms for a single analytical run. The two columns in series are packed with two different stationary phases. Furthermore, this system is equipped with two UV detectors set to two different wavelengths (255 nm and 275 nm), so the analytes that coelute in the first column can be separated on the second; compounds less retained on the first column could be more retained in the second and vice versa. The usefulness of the dual column configuration is exemplified in Figure 5.4. In fact, by plotting the LRI values calculated on the second trace (LRI 2), which takes into account the retention on both columns, against the LRI values on the first column (LRI 1), a bidimensional map is obtained. Despite the compounds are mainly arranged along the main diagonal, some critical pairs were successfully discriminated thanks to the coupling of different retention mechanisms: the critical pairs of

compounds 11 and 12 and compounds 22 and 23 can be reliably identified thanks to significantly different LRI values on the biphenyl column (different values for LRI 1); on the other hand, compounds 2 and 4, 8 and 10, 18 and 19, and compounds 11, 13 and 14 have the same LRI on the biphenyl column and different values for LRI 2. For the sake of clarity, Figure 5.5 shows the analysis of 3 cannabinoids, including the critical pair of compounds 2 and 4, on the dual column setup.

In Figure 5.4 insert A, which is an expansion of the 2D graph in the regions of LRI 1510 to 1540, the distinction between compounds 11, 13 and 14 is better illustrated. In particular, if the difference in a given parameter is higher of the total of their standard deviations, two compounds can be recognized consistently [37]. As a result, system repeatability is very important. Figure 5.4 and its inserts show error bars for each point to clearly highlight possible mismatching between adjacent peaks, even when the standard deviation (obtained from 4 repetitions at 2 concentration levels) of each measurement. The only superimposition is between compounds 20 and 21. However, for further discrimination, the absorbance ratio on the two detectors can be used as a third parameter. The absorbance ratios of compounds 20 and 21 to LRI 1 and LRI 2, respectively, are shown in inserts B and C of Figure 5.4, indicating that they cannot be reliably identified based on both LRIs, but their ratios of absorbance are significantly different (the absorbance ratio of compound 21 is double that of compound 20), allowing for discrimination.

Therefore, when the single column technique fails, the three filter database, obtained using a dual column setup, could be used to identify cannabinoids in cannabis inflorescences.

However, due to the low molar absorption capacity of cannabinoids, the observed results must be considered preliminary because the absorption wavelengths used do not allow the sensitive determination of real samples. To overcome this limitation, a detector with a lower wavelength could be used in the near future.

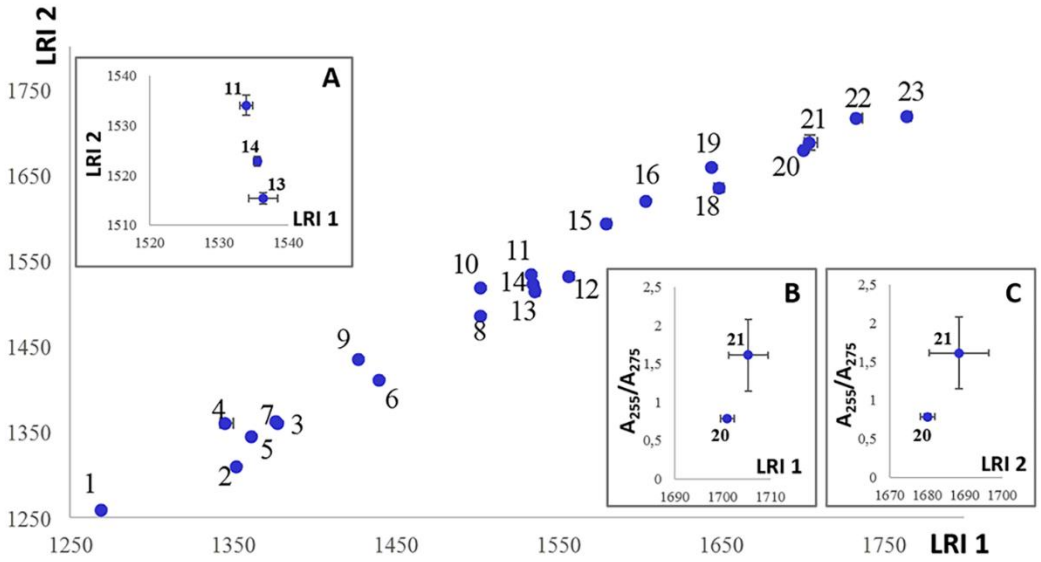

Figure 5.4. *Plot of LRI 2 against LRI 1; enlargement of LRI regions 1510-1540 in insert A; absorbance ratios of compounds 20 and 21 against LRI 1 and LRI 2 in the inserts B and C, respectively. For peak numbering, see Table 5.1.*

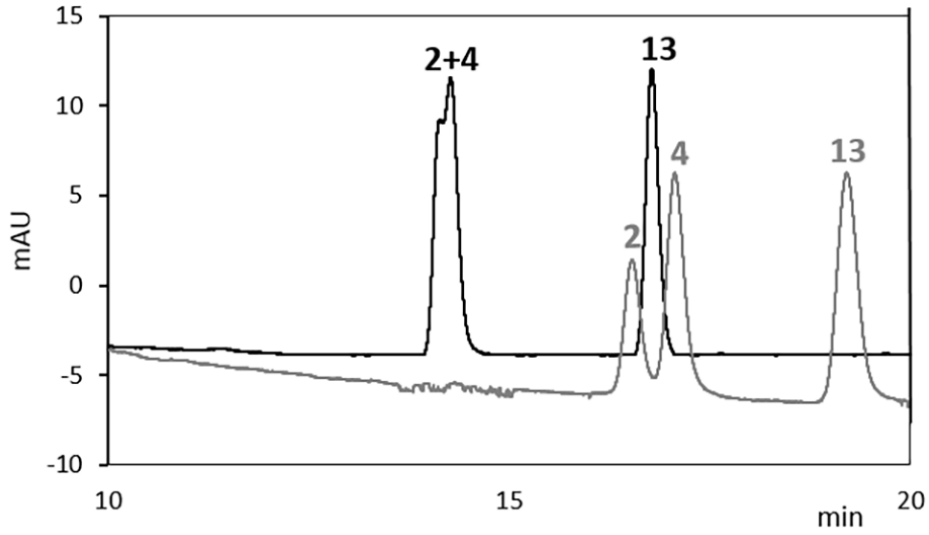

Figure 5.5. *Separation of a standard mixture on the dual column setup (— first column C8, 10 cm×150 μm, 3μm dp, detection at 255 nm; — second column biphenyl 5 cm×150 μm, 3μm dp, detection at 275 nm). For peak numbering, see Table 5.1.*

5.4. Assessment of the greenness profile.

The AGREE software, freely available online, automatically assigned a score for each of the 12 GAC principles, as established by Galuszka et al. [27]. According to this procedure, the maximum value of 1 is assigned to the greenest process, while 0 is assigned to a totally not green process. In order to evaluate the potential of the hand-portable analytical device for on-site analyses, the following scores were assigned to each variable:

1. Sample treatment: the method is based on an in-field sampling and online analysis, which avoids sample collection, preservation, transport and storage. The assigned score is 0.78.
2. Sample size: 0.5 g of sample was used. The assigned score was 0.75.
3. Analytical device location: the employed hand-portable Cap-LC setup allows for in-situ analysis. The assigned score was 1.
4. Sample preparation steps: the extraction procedure simply involves grinding, solvent extraction and solvent evaporation as main steps. The assigned score was 1.
5. Automation of the method and miniaturization of the sample preparation procedure: since no automation is present in any step of the analytical procedure and sample preparation was not miniaturized, the assigned score was 0.
6. Derivatization: since no derivatization was required, the assigned score was 1.
7. Analytical waste: the main contribution to waste production was represented by the extraction volume, while the waste of the Cap-LC instrument can be considered negligible. The assigned score was 0.26.
8. Multianalyte methods and analytical throughput: 24 analytes can be determined in a single run and 2 samples can be analyzed in 1 hour. The assigned score was 0.89.
9. Energy consumption: of the hand-portable instrument is equipped with a 0.125 kWh battery power that allows for 10 hours analyses without recharging. The assigned score was 1.
10. Source of reagents: bio-based ethanol was used for the extraction. The assigned score was 0.5.
11. Solvent and reagent toxicity: since a miniaturized LC method, which employs acetonitrile and 0.1% TFA, was developed, the assigned score was 0.8.
12. Safety: the only considered hazards were the high flammability for acetonitrile and the corrosivity of TFA, while the small employed volumes do not make the method toxic for humans or aquatic life. The assigned score was 0.6.

The obtained circular diagram is reported in Figure 5.6. It shows in a color scale the 12 scores, from red for score 0 to dark green for score 1, and in the middle the average score.

It can be concluded that a green method was developed for the in-situ analysis of cannabinoids in hemp inflorescences. However, the analysis of the diagram clearly suggests that sample preparation should be miniaturized to better fulfill principle 5 and 7.

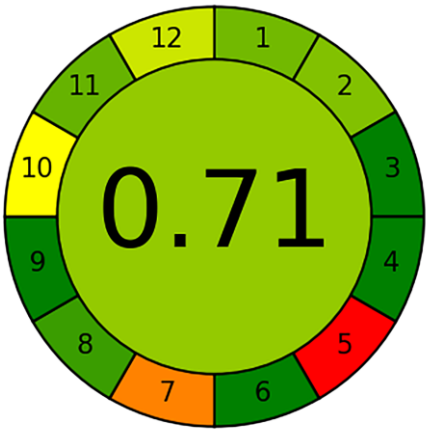

Figure 5.6. *Results of AGREE analysis for the on-site Cap-LC method for the determination of 24 cannabinoids in the extract of hemp inflorescences.*

5.5. Conclusion

The present research proposes the use of an innovative instrumental setup based on a hand-portable miniaturized LC system for the *in-situ* screening of non-psychoactive cannabinoids in inflorescences of *Cannabis sativa* L. A cap-LC method was developed for the efficient separation of 24 cannabinoids and their reliable identification through the LRI approach, recently implemented in LC and for the first time applied to this kind of analytes and instrumentation. The use of a dedicated software made fully automatic the identification process, thus reducing the total analysis time. Future perspectives could regard the coupling of the cap-LC system with a more sensitive and selective detector, such as photodiode array or even mass spectrometry, to add qualitative information helpful for the identification of novel molecules (e.g., degradation products) and make the method suitable for the analysis of different real-world samples, even considering forensic applications which could require the determination of cannabinoids and/or their metabolites in biological fluids, even if they are present at low concentrations.

†This chapter has been adapted from the following publication: R. La Tella, F. Rigano, P. Guarnaccia, P. Dugo, L. Mondello in "*Non-psychoactive cannabinoids identification by linear retention index approach applied to a hand-portable capillary liquid chromatography platform*" Anal. Bioanal. Chem. (2022) DOI: 10.1007/s00216-021-03871-x.

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Chapter 6

Development of a prototype system based on High Temperature Liquid Chromatography for the determination of parabens in cosmetic and food samples

6.1. Introduction

Parabens are esters of p-hydroxybenzoic acid; these compounds are chemicals commonly used as artificial preservatives in various products, like foods, personal care products, pharmaceutical drugs, and beverages. [1,2]

The most commonly used parabens are methyl- (MeP), ethyl- (EtP), propyl- (PrP), and butyl-parabens (BuP).

The high antibacterial activity in a wide pH range, the low cost, the thermostability, the odorless, the tasteless, have attacted the interest of researchers for these compounds [3]. However, both *in vitro* and *in vivo* studies have shown that these substances, used since 1920 in cosmetics and pharmaceuticals, interfere with the endocrine system. [4,5]

Currently, the legal provision concerning this field is the Commission Regulation (EU) No 1004/2014 on cosmetic products that sets a maximum limit of 0.4% and 0.8% for single esters and mixtures of esters, respectively. [6]

Regarding parabens in food, only the use of MeP, EtP and their sodium salts have been assessed by the European Food Safety Authority (EFSA). EFSA has established a group ADI (acceptable daily intake) of 0-10 mg / kg body weight (milligrams / kilogram body weight) for these molecules [7]; PrP cannot be included in this group due to a lack of studies evaluating its safety. Indeed, it has been shown that as the alkyl chain increases, so does the antimicrobial activity and estrogenic activity. For example, on the production of spermatozoa in young male rats, PrP and BuP have shown the greatest toxic effects. [8,9].

Until now, HPLC-DAD, LC-MS and GC-MS methods have been widely applied for the analysis of these compounds in cosmetics and food samples. [10]

To this regard, separation techniques, including mainly LC, involves the consumption of large amounts of organic solvents. The use of organic solvents and the production of hazardous waste production are a worldwide problem that has being influenced not only human health but also our environment. In LC, an ideal mobile phase should possess many requirements, such as: the ability to dissolve ionized, polar and nonpolar analytes; no flammability; nontoxicity; high grade of purity; low viscosity to generate low backpressure; high diffusion rate; and low cost. Most organic solvents fail on a number of these criteria, especially low cost, toxicity and flammability.

This issue can be overcome by replacing the organic solvent with pure water at high temperature, making more eco-sustainable the developed analytical method.

The pioneering research was conducted by Smith et al. in 1990. In this study, pure water as a mobile phase was used in reverse phase liquid chromatography at temperatures between 100 and 200 ° C. [11]

Pure water at elevated temperature can be used as a mobile phase in LC mainly due to the variation of the dielectric constant. In fact, as the temperature increases, the dielectric constant of water decreases, becoming similar to that of water-methanol or water-acetonitrile mixtures at room temperature. [12] This allows the separation of many classes of compounds, both polar and nonpolar. The derive technique is called "High Temperature Liquid Chromatography" (HTLC) or "SuperHeated Water Chromatography" (SHWC)

In the past, Dugo and co-workers determined the presence of parabens in a cosmetic sample using this analytical technique. In their research they used a ZR-Carbon C18 column and the best results were obtained at 170 ° C using water/tetrahydrofuran (95:5 v/v). [13] The main reason that prompted us to re-propose and validated this analytical method was the growing interest in green approaches. For sure, the use of tetrahydrofuran should be avoided, due to its high flammability, toxicity and suspected carcinogenicity.

The four crucial points of HTLC or SHWC are: 1) the need of stable stationary phases at high temperatures, 2) ensuring efficient heating of the mobile phase before entering the analytical column, 3) maintaining water in the liquid state from injector to detector, and 4) the need to use a post-oven cooling system since most of the detectors cannot be used at high temperatures.

As for LC columns resistant to high temperatures Zirconia [13] or polymer-based phases [11,14] were previously used, but they were unstable at temperatures above 200 ° C. Recently, porous graphitic carbon (PGC) has been proposed as an alternative to polymer-based phases, as it provides significant retention for both non-polar and polar analytes. [15] In this work, the performance of a carbon graphite columns was evaluated. An HPLC system was interfaced with a GC oven, which the LC column was located; to obtain a rapid and efficient heating of the eluent coming from the autosampler before entering the column, a preheating tube was placed inside the GC oven between the outlet of the autosampler and the inlet of the column. Finally, a stainless steel restrictor tube was connected the column to a cooling loop and the cooling loop was connected to the restrictor to cool the LC effluent before entering the UV detector.

The extraction procedure was fast and environmentally friendly. In fact, only water and ethanol were used as extraction solvents. In this regard, compared to MeOH or ethyl acetate used in other research methods [16,17], ethanol was classified as green solvent both for its limited toxicity, and because can be produced from biomass in a renewable way. [18]

The aim of this work is to show the recent developments of the HTLC technique according to an eco-sustainable approach.

6.2. Experimental

6.2.1. Chemicals and reagents

LC-MS ethanol and water, methyl paraben, ethyl paraben, propyl paraben and butyl paraben were purchased by Merck Life Science (Darmstadt, Germany). Standard solutions were prepared by mixing the four standards up to a total concentration of 250 ppm each.

6.2.2. Sample and sample preparation

A candy sample and a blush sample were purchased from a local market. The samples were in solid form and were crushed and homogenized in a mortar prior to analysis.

For parabens extraction, about 0.5 g of solid samples were weighed and dissolved in 3 mL of hot water (45 ° C) and 2 mL of EtOH. The samples were sonicated for 2 minutes and subsequently filtered by passing through 0.45 μm membrane filters before being injected into the SHWC system.

6.2.3. Instrumentation and chromatographic conditions

The analyses were performed using a Flexar HPLC system (PerkinElmer Inc., Waltham, MA, USA) equipped with a photo diode array detector (Flexar PDA Plus Detector), a quaternary LC pump (Flexar quaternary LC pump) and an autosampler (Flexar LC autosampler).

The LC column was placed inside MasterGC oven (PerkinElmer Inc., Waltham, MA, USA). The original Perkin Elmer 0.15 mm × 43 cm ID×L connection was used from the autosampler to preheater tube, of which 9 cm was inside the oven. The preheater tube, i.e. a stainless steel tube 0.13 mm × 52 cm ID×L, was placed inside the oven and was used for an efficient mobile phase heating prior to enter into the column. A stainless steel tube (0.13 mm × 140 cm ID×L) was used from the column outlet to the cooling loop. The cooling loop was a stainless steel tube 0.5mm × 56 cm ID×L. A PEEK tube (0.13 mm × 62 cm IDxL) was placed between the cooling loop and the PDA cell.

The cooling of the mobile phase was achieved by using a liquid CPU cooler (CORSAIR Hydro Series H150i PRO), which includes a 360mm radiator for noise-free cooling. Corsair iCUE software allows to adjust individual fan speeds and pump speeds by monitoring CPU and coolant temperatures. Figure 6.1 reports the picture of the instrument, while Figure 6.2 A reports the picture of the cooling system, connected to the PC for the monitoring of the temperature (Figure 6.2 B).

The chromatographic column was a PGC column 50 × 3.0 mm , 2.7 μm L×ID provided by Merck Life Science (Darmstadt, Germany).

The LC conditions were: water and ethanol as the mobile phase. Injection volume was set at 1 μL and the sample was diluted to 250 ppm prior of the analysis. Wavelength range of PDA was 190-400 nm,

sampling frequency 6.25 Hz. Flow rate, oven temperature and mobile phase composition were changed during column testing.

Figure 6.1. *Instrument.*

A

B

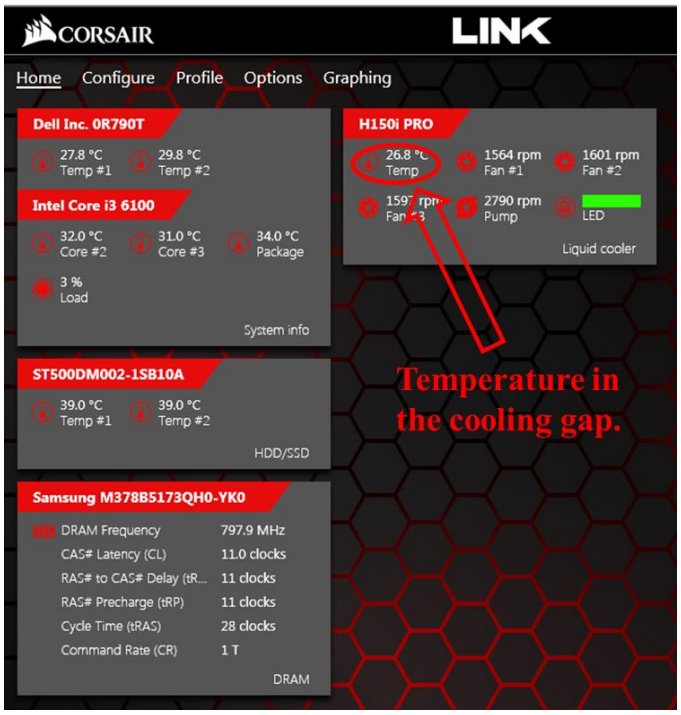

Figure 6.2. *(A) cooling gap (56 cm × 0.5 mm ID stainless steel tube), (B) software (Corsair Link) window.*

6.3. Results and Discussion

6.3.1. Development of HTLC method

In this study, the goal was to achieve an optimal separation of a mixture of four parabens on a porous grafitic carbon stationary phase using water with a small percentage of ethanol as mobile phase.

The crucial point of the SHW technique is the maintenance of the mobile phase in the liquid state. In fact, at temperatures above 100 ° C at standard atmospheric pressure, the water boils.

As the pressure increases, the boiling point also increases. Considering the high operating temperatures, which reduce the viscosity of the water, it is necessary to apply a high back pressure and a high flow rate to keep it in a liquid state, thus also increasing the analysis speed. For these reasons, it is necessary to use a back pressure restrictor. Our instrumental prototype is equipped with a restrictor stainless steel tube, which is cheaper than a back pressure control module, placed between the column outlet and the cooling circuit. The final length of this tube was optimized and evaluated based on chromatographic analyses performed under different temperature and flow rate conditions.

The pumping system consists of two high pressure pump. This allows use of high flow rate as with conventional HPLC instrumentation.

The packaging material, PGC, can withstand temperatures of 250 ° C.

Different temperatures were tested, but the shortest analysis times, while maintaining satisfactory efficiency and resolution, were obtained at a temperature of 250 ° C.

Preliminary analyses at temperatures below 100°C, using 40%, 60% or 80% of organic solvent (EtOH), gave reasonable analysis times, but our aim was to reduce the use of ethanol. Using 200 ° C, at flow rate 1.2 ml/min and 30% of EtOH, the first and second compound co-eluted, as shown in figure 6.3.

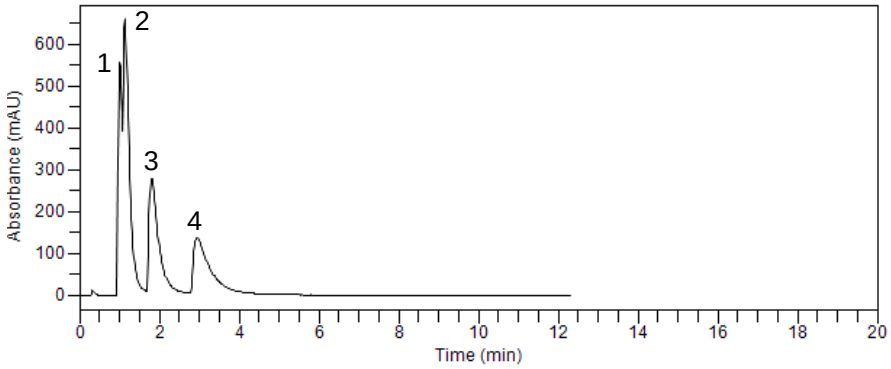

Figure 6.3. *Parabens separation on the carbon column 50×3.0 mm LxID, at a flow rate of 1.2 mL/min, 200 °C, water/ethanol (70:30 v/v). Peak legend: 1) methylparaben, 2) ethylparaben, 3) propylparaben, 4) butylparaben.*

It was therefore necessary to increase the temperature to allow the separation. The mobile phase composition is the most important parameter that plays a significant role in the chromatographic separation. Unfortunately, at 100% water as mobile phase, the chromatographic parameters are not good; asymmetric and not well resolved peaks are obtained. Under optimized conditions, using 250 ° C and water with 10% EtOH as the mobile phase, the parabens were separated in about 6 min, as shown in Figure 6.4. Another parameter optimized was the flow rate; for the porous graphitic carbon phase, the optimal flow rate for the separation of a mixture of parabens is 1.2 ml/min for columns 50 × 3.0 mm, 2.7 μm L×ID (typical flow rates vs column ID are 0.6 mL/min for optimal efficiency according to the van Deemter equation). Gradient elution allowed for better separation of all compounds, especially the increase in organic solvent in the last 6 minutes of analysis it allows a lower retention of the third and fourth compounds, therefore a higher resolution and efficiency. The separation of parabens was obtained on a PGC column 50 × 3.0 mm L × ID using the following gradient: 0-4 min 90-10% H_2O/EtOH, 4-10 min 70-30% H_2O/EtOH as shown in Figure 6.5. For these considerations, the analyses of the real world samples were performed in gradient mode.

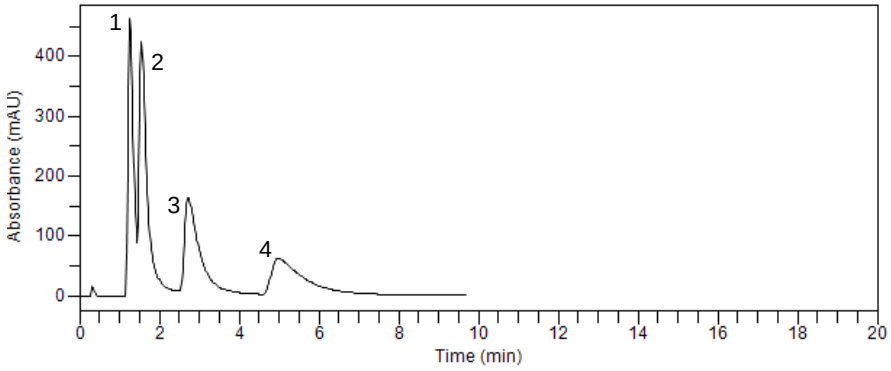

Figure 6.4. *Paraben separation on the carbon column 50×3.0 mm LxID, at a flow rate of 1.2 mL/min, 250 °C, water/ethanol (90:10 v:v) . Peak legend: 1) methylparaben, 2) ethylparaben, 3) propylparaben, 4) butylparaben.*

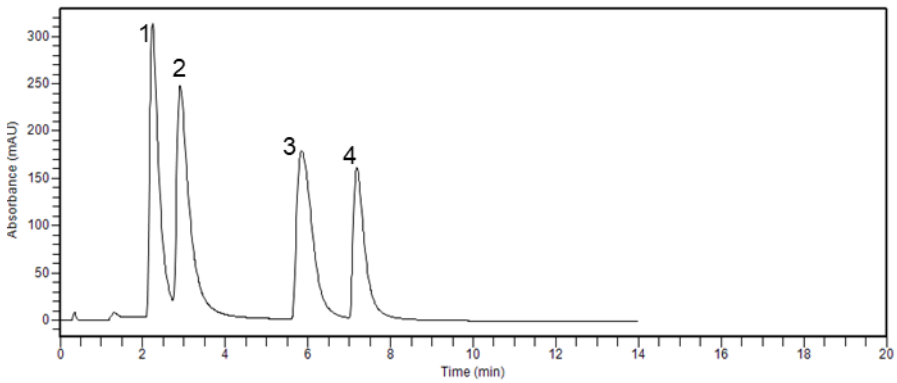

Figure 6.5. *Parabens separation on the carbon column 50×3.0 mm LxID, at a flow rate of 1.2 mL/min, 250 °C, water/ethanol (0-4 min 90/10%, 4-10 min 70/30%). Peak legend: 1) methylparaben, 2) ethylparaben, 3) propylparaben, 4) butylparaben.*

6.3.2. Paraben concentrations in food and cosmetic samples

To evaluate the optimized method, a candy sample and a blush sample were analyzed. Samples were extracted as reported in section 6.2.2. The chromatogram obtained from a candy sample using H_2O/EtOH at 250 ° C is shown in Figure 6.6. (A). Methyl-paraben is identified, which has the same retention time shown in the analyzes performed under the gradient mode. Figure 6.6 (B) shows the chromatogram obtained from the blush sample. All four parabens were identified in this sample.

Compared to the separation of parabens extracted from the body cream obtained by Dugo and co-workers [13], this method is more fast and eco-friendly.

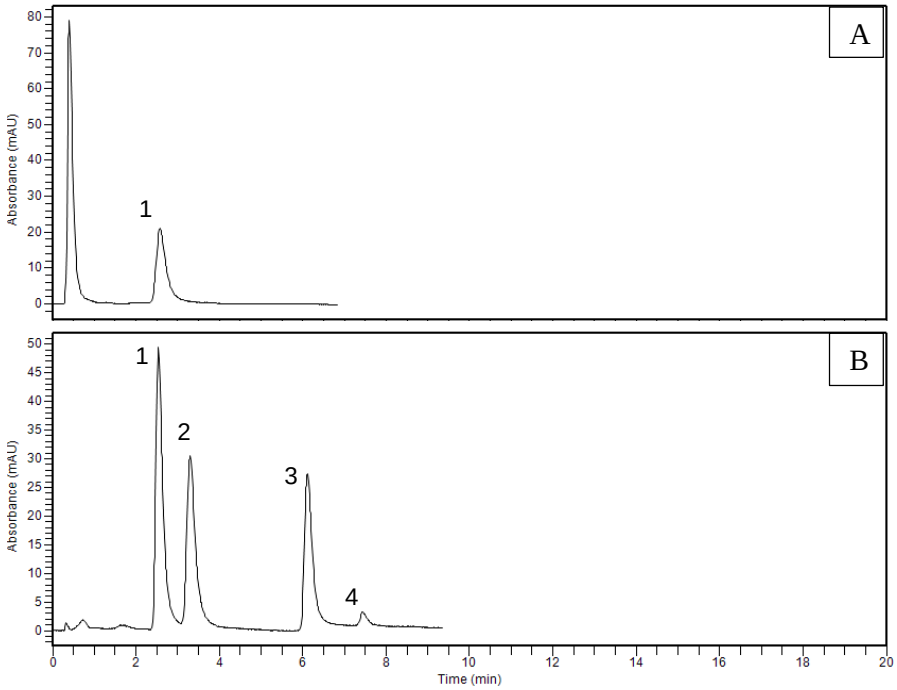

Figure 6.6. *(A) Parabens in candy sample, at a flow rate of 1.2 mL/min, 250 °C, water/ethanol (0-4 min 90/10%, 4-10 min 70/30%). (B) Parabens in blush sample, at a flow rate of 1.2 mL/min, 250 °C, water/ethanol (0-4 min 90/10%, 4-10 min 70/30%). Peak legend: 1) methylparaben, 2) ethylparaben, 3) propylparaben, 4) butylparaben.*

6.4. Conclusion

The present research proposes the use of an instrumental prototype based on the use of superheated water for the simultaneous determination of four parabens in food and cosmetic samples.

The fast, simple and eco-sustainable sample preparation technique, only by direct extraction with ethanol and hot water, makes this method easy to use.

The method developed in this research may be of potential interest for the analysis of preservatives through a "green" approach.

Further studies will be carried out for the validation of the method according to Eurachem guidelines.

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Chapter 7

Evaluation of the level of toxic contaminants and essential molecules in the context of the re-use of tuna fishery industry by-products†

7.1. Introduction

The Circular Economy (CE) is an economic model based on resource reuse, which involves abandoning the practice of disposables and implementing a successful recycling plan [1]. Gunter Pauli, a Belgian economist, used the term "Blue Economy" to describe a branch of CE that focused on maximizing the use of aquatic resources rather than on new sources of investment [2]. Besides the economic benefit, such an approach aims to be fully eco-sustainable, preserving the environment. In fact, Pauli thesis is based on the observation that in nature there is no waste production. The indiscriminate use of resources required by human activity in the last century caused an increasing pressure on the marine ecosystem, forcing us to reuse what already consumed as a source of new wealth [2]. In comparison to the Green Economy, which aims to improve production processes using renewable energy, environmentally-friendly materials and reducing the use of toxic chemicals, the Blue Economy focuses on the recycling of fishery products, allowing biodiversity to evolve while maintaining the natural balance of the marine ecosystem [3]. According to this concept, everything that represents a waste of a production cycle can be a primary resource for a new one. The tuna processing industry generates a significant quantity of waste since less than 50% of the raw material is destined for human consumption [4], such as canned foods. Even when fresh, more than half are rejected or thrown back into the sea, altering the ocean ecosystem and causing the loss of high-value biomolecules like bio-peptides, omega-3 fatty acids, essential amino acids, essential metals, and vitamins. Fish is considered a functional food thanks to the synergic effect of all these components, which can contribute to reduce chronic diseases and improve overall well-being, in both therapeutic and preventive approaches [5]. On the other hand, by focusing on metals or amino acids, tuna, which is a large pelagic species belonging to the Scombridae family, is considered potentially harmful due to its level of heavy metals (e.g. mercury), proportional to its size, and the high concentration of histidine, which could be converted into histamine. Histamine is a biogenic amine that is primarily involved as a mediator of anaphylactic or allergic reactions. Mast cells and basophil granulocytes are produced and stored in the human body, than released when these immune cells come into contact with an allergen to which the body has previously become sensitized. Once released, it interferes with different physiological functions, causing vasodilation, increased vascular permeability, bronchoconstriction, and mucus secretion [6]. Histamine intoxication is caused by eating foods with high amounts of histamine; this intoxication is commonly known as scombroid syndrome, since it happens more frequently after eating scombroids (e.g. tuna and mackerel) that have been stored incorrectly [6,7]. The maximum production of histamine occurs at 27-28 ° C and stops at temperatures ≤ 6 ° C, so it is important to keep the fish at low temperatures, since histamine does not degrade with cooking, freezing, canning or smoking. Histamine has no smell or taste, therefore poisoning can occur

without the deterioration of the fish being perceptible from an organoleptic point of view [8]. Only a laboratory test can indicate whether or not there are hazardous amounts present. The recommended amount of HA in fish species, according to the Commission Regulation (EC), No 2073/2005, must not exceed 200 mg/kg and 200–400 mg/kg in the fishery products [9]. While many studies have been published in the literature regarding the quantification of several heavy metals in both fresh and canned fish [10-12], as well as several methods for monitoring histamine in fish products [13-15], the goal of this research was to consider the waste as a potential source of essential metals and amino acids, carefully monitoring the amount of toxic heavy metals and histamine, and comparing the results with the levels found in the edible part. Recently, it was shown that the omega-3 concentration of tuna fish waste products is higher than the edible part [16], an interesting result that could lead to the production of supplements and nutraceuticals employing waste as starting material, encouraging a completely eco-sustainable approach. Since the definition of functional food or components naturally implies the absence of any harm to human health, heavy metals and histamine must be determined as control points in the strategy mentioned above. As for metals, we used Inductively Coupled Plasma–Mass Spectrometry (ICP-MS) to assess the metal content, which includes both heavy (e.g. As, Cd, Hg, and Pb) and essential metals (e.g. Se, Mn, Cu). In the case of heavy metals, there is a legislation for Pb, Cd and Hg, with limit of 0.30 mg kg^{-1}, 0.10 mg kg^{-1} and 1.00 mg kg^{-1}, respectively, in the muscles of numerous fish, including Thunnus species [17]. The same regulation specified maximum limits for the same metals in dietary supplements of 3 mg Kg^{-1}, 1 mg Kg^{-1} and 0.1 mg Kg^{-1}, for Pb, Cd and Hg, respectively. These elements accumulate along the food chain (bioaccumulation), so they must be controlled at every step of the food supply chain. For example, the maximum content of these metals in fish meals used as feed for both terrestrial and aquatic animals is regulated by Commission Regulation EU 574/2011 [52], because, despite the immediate damage to the animals, they and the derived food products (e,g. eggs) will be consumed by human increasing the total intake of these toxic metals. In this case, a limit is also set for arsenic, most likely to avoid bioaccumulation problems. As exposure research revealed significant levels of inorganic arsenic in high consumers of rice, arsenic is currently limited only in rice and derived products [18]. Similar results were not obtained when fish and seafood consumption were considered, because arsenic occurs in these foods mainly in the organic form, namely arsenolipids, arsenoproteins, arsenosugars, and arsenobetaine, to which major efforts should be directed to produce speciation data in different foodstuffs and elucidate their metabolism in the human organism [19,53]. The Commission Regulation CE 1170/2009 [54] establishes the mineral forms that can be added to food and food supplements, in line with the maximum recommended daily intakes for essential metals. As a result, in order to avoid toxicity problems, their evaluation is required during the production of food supplements [20]. As for free

amino acids (FAAs) and hystamine, we validated a fast-gas chromatography/mass spectrometry (Fast-GC/MS) method for the analysis of a total of 29 species, which included all the essential AAs and histamine (HA). To convert the polar FAAs into more volatile, GC-amenable species, the method includes a rapid derivatization procedure. One of the most frequent derivatization approach is silylation, which uses reagents like N-MethylN-(trimethylsilyl) trifluoroacetamide (MSTFA) and its derivatives to obtain highly volatile derivates [21]. However, not all of them are stable; for example arginine decomposes to ornithine [22], whereas glutammic acid rearranges to pyro-glutammic acid. Furthermore, both the derivatizing agent and the derivatives are sensible to moisture, making sample handling difficult. According to Kritikos et al. [23], the derivatization used in this study, used propyl chloroformate as derivatizing agent. The ability to work directly in aqueous solution [24] a the rapidity of the process (2 min at room temperature vs. a minimum of 20 min at 60-70 °C for the silylation) are the main advantages of this strategy. The use of a GC separation, rather than a liquid chromatography (LC) method in the chromatographic approach has made it possible to benefit from a fully automatic identification procedure, thanks to the coupling with electron ionization MS (EI-MS) detection. As it is well-known, EI-MS can produce highly reproducible spectral data that is unaffected by matrix interferences and operating parameters (pressure, temperature, solvent), and can therefore be matched to thousands of spectra present in commercially available spectra [55,25]. Furthermore, GC has a higher retention data reproducibility, mainly due to the negligible influence of the mobile phase, so that retention data in the form of the well-established Linear Retention Index (LRI) parameters to be combined with MS data in a dualfilter database to obtain univocal and highly reliable identification in a fully automatic way [26,27]. Consequently, the developed method could be successfully employed for routine analysis of both FAAs and histamine quantification due to the reduced total analysis time (from sample preparation to data processing). Moreover, it could be potentially used for protein amino acids quantification following total protein hydrolysis. The quantification of FAAs, such as essential metals, is necessary to ensure compliance with the recommended dietary intakes [28]. To the author's knowledge, the tuna waste products, which are frequently used in the production of fish meals used as feed, have never been characterized for their content of metals or amino acids, including both toxic and essential compounds. In this context, the technique presented here should be used to monitor these compounds in all the steps of the waste recycling process, in order to optimize the yield of bioactive compounds, while reducing pollulant quantities.

7.2. Experimental section

7.2.1. Chemicals

Suprapur nitric acid (HNO_3 > 69% (v/v)), HPLC grade chloroform ($CHCl_3$), methanol (MeOH), isooctane and n-propanol, 3-picoline, propyl chloroformate, sodium hydroxide (NaOH), and hydrochloric acid (HCl) were all purchased from Merck Life Science (Merck KGaA. Darmstadt, Germany). Ultrapure water (18 MΩ-cm) was generated though the Milli-Q advange A10 system (Merck KGaA). Standard stock solutions containing 1000 mg L^{-1} of As (arsenic), Cd (cadmium), Cr (chromium), Cu (cupper), Mn (manganese), Ni (nickel), Hg (mercury), Se (selenium), Sr (stronzium), Pb (lead), V (vanadium) and Zn (zinc), and internal standard solutions containing 1000 mg L^{-1} of Sc (scandium), Y (yttrium), In (indium), Rh (rhodium) and Ho (holmium) were purchased from Merck Life Science. A multi-element solution (CPA Chem, Stara Zagora, Bulgaria), composed by Be (beryllium) 1000 μg L^{-1}, Co (cobalt) 500 μg L^{-1}, Mn (manganese) 500 μg L^{-1}, Bi (bismuth) 200 μg L^{-1}, In (indium) 200 μg L^{-1}, Ce (cerium) 200 μg L^{-1} in HNO_3 1%, was used for daily instrument calibration. Ultra-pure grade carrier and collision gas (Argon (Ar) 99.99% pure and Helium (He) 99.9996% pure, respectively) were supplied from Rivoira S.r.l (Messina, Italy). Certified Reference Material (CRM) ERM®-CE278k (mussel tissue), ERM®-BB422 (fish muscle) and ERM®-CE464 (tuna fish) were acquired from the Institute for Reference Materials and Measurement of the Joint Research Centre (JRC) of the European Commission (Geel, Belgium). A certified fish oil sample (PronovaPure, Ludwigshafen, Germany) in which the metals of interest were not detected was used as sample blank for the calculation of limit of detection (LOD) and quantification (LOQ). Analytical standards of alanine (ALA), sarcosine (SAR), glycine (GLY), α-aminobutyric acid (ABA), valine (VAL), β-aminoisobutyric acid (BAIB), norvaline (NVA, Internal Standard, IS), leucine (LEU), alloisoleucine (AILE), isoleucine (ILE), threonine (THR), serine (SER), proline (PRO), asparagine (ASN), thiaproline (TPR), aspartic acid (ASP), methionine (MET), proline, 4-hydroxy (HYP), glutamic Acid (GLU), phenylalanine (PHE), α-aminoadipic acid (AAA), α-aminopimelic acid (APA), glutamine (GLN), histamine (HA), ornithine (ORN), proline, glycyl (GPR), lysine (LYS), histidine (HIS), tyrosine (TYR), and tryptophan (TRP), were purchased from Merck Life Science.

7.2.2. Samples and sample preparation

Tuna fish samples (n=3) were acquired in a local fish store in Messina (Sicily, Italy) during the period September 2018 - July 2019. Both the edible fraction (muscle) and waste parts (skin, heart head, guts) were provided. Industrial wastes from tuna fish processing were supplied from an Italian fish company in November 2019. Raw and cooked homogenized waste tissues, the derived lipid fractions (the oils), aqueous fractions and their protein solid fractions were provided. The raw waste contains the parts

immediately removed from the fresh fish and are composed mainly of guts, tails and heads. The remaining part was cooked to facilitate the removal of skin, fishbones and dark muscles, which compose the cooked waste. Finally, the oils and the aqueous and protein solid fractions were industrially obtained from the cooked wastes (also the raw one was cooked to improve the separation process), pressed and decanted. In parallel, the well-established Bligh and Dyer extraction method [29] was performed on fresh tuna tissues in order to obtain the oil fractions, the aqueous fractions and the protein solid fractions at the laboratory scale. Briefly, 10 g of homogenized sample were placed in a flask, added with 30 mL of a $CHCl_3$/MeOH 1:2 (v/v) mixture and kept under magnetic agitation for 10 min. Afterward, 20 mL of a $CHCl_3$/water 1:1 (v/v) mixture were added and further agitated for 1 minute. The mixture was centrifuged for 15 min at 3000 rpm. The lower chloroform phase was transferred to a flask, while the polar phase was extracted again with 20 mL of 10% (*v/v*) methanol in chloroform. Then, the two chloroform phases were pooled together and evaporated at 30 °C by using a rotary evaporator (Hei-VAP Precision, Heidolph), thus obtaining an oil fraction. The oil, the methanolic/aqueous phase, containing the FAA fraction, and the solid residue, containing mainly proteins, were all stored at -20 °C until metal and FAA analysis.

7.2.2.1 Sample preparation for metal analysis

All samples were weighed (0.5 g for all analyzed samples, with the exception of 0.2 g for the oils) and placed in a Teflon digestion vessel with 10 ml of Suprapur concentrated HNO_3 to undergo mineralization through the MARS 6 One Touch Technology Microwave lab station (CEM Microwave Technology Ltd., North Caroline, USA. Microwave program was as follows: 25–200 ° C in 25 min at 1800 W (hold for 15 min). After cooling, digests were diluted with deionized water in a 50 mL volumetric flask, and subjected ICP-MS analysis. A standard mixture containing 10 mg L^{-1} of each metal were prepared daily by dilution of 100 μL of each standard stock solution in 2% (*v/v*) HNO_3 up to a final volume of 10 mL. Working standard mixture solutions at concentrations of 0.5 μg L^{-1} , 1 μg L^{-1}, 2 μg L^{-1}, 4 μg L^{-1}, 8 μg L^{-1}, 15 μg L^{-1}, 30 μg L^{-1} were prepared by further dilutions and used for the daily building of calibration curves. A 2% (*v/v*) HNO3 solution and the certified fish oil sample were selected as reagent blank and sample blank to calculate instrumental and method limits of detection and quantification, respectively. Specifically, both were spiked at a metal concentration of 0.1 ppb and subjected to direct ICP-MS analysis in the case of reagent blank and mineralization and ICP-MS analysis in the case of the sample blank.

7.2.2.2. Sample preparation for the analysis of Free Amino Acids

One hundred µL of the aqueous phases coming from the Bligh and Dyer extractions or provided from the industry were added with 100 µL of IS solution; the pH of the solution was raised by adding 120 µL of 0.33M NaOH solution and 50 µL of 3-picoline in propanol (1:4, *v/v*). Fifty µL of propyl chloroformate in chloroform/iso-octane (2:6:2, *v/v/v*) were added to the sample and the solution was mixed for 1 min (2000 rpm), equilibrated for 1 min and mixed again for 1 min (2000 rpm). The derivatized amino acids were extracted by adding 100 µL of a mixture isooctane/chloroform (9:1, *v/v*) and vortexed for 1 min (2000 rpm). After decantation the upper organic phase was recovered and evaporated under a gentle stream of Nitrogen, and the derivatized amino acids were re-dissolved in 100 µL of a mixture isooctane/chloroform (8:2, *v/v*). If necessary the samples were properly diluted to guarantee their fitting in the linearity range of the calibration curves. A standard mixture containing 200 nmol mL^{-1} of each analytical FAA standard was prepared by weighing the proper amount of each FAA, diluted with 20 mM HCl aqueous solution. Working standard solutions at concentration of 10, 20, 50, 100, 150 were prepared by further dilutions. All standard mixture solutions were subjected to the same sample preparation procedure prior of the analysis for the building of calibration curves.

7.2.3. Instruments and analytical conditions

ICPMS-2030 Inductively Coupled Plasma–Mass Spectrometry (Shimadzu, Duisburg, Germany) equipped with AS-10 autosampler (Shimadzu Deutschland GmbH, Duisburg, Germany) was used for metals analyses. Instrumental parameters are summarized in Table 7.1. The LabSolution ICPMS software, version 1.12, was used for data acquisition and processing. A GCMS QP2010 Ultra (Shimadzu, Duisburg, Germany), equipped with a AOC-20i autosampler, was used for GC-MS analyses of derivatized amino acids. Injections were performed in split mode (15:1); the injection volume and injector temperature were 2 µL and 300 °C, respectively. Separations were achieved by using a SLB-PAHms 10 m × 0.25 mm × 0.25 µm custom column obtained from Merck and helium as gas carrier at a constant linear velocity of 67.9 cm/s (initial inlet pressure 6.6 KPa), under the following temperature program: 30°C/min, from 110°C to 320°C (total analysis time 7 min). MS conditions were as follow: source temperature 240°C, interface temperature 200°C, event time 0.1 sec, mass range 45-450 *m/z*. The GCMSsolution software (version 4.41) was used for data acquisition and processing. A homemade spectral library with embedded LRI data was built by standard injection of 30 amino acid derivatives and used for the reliable identification of unknown samples.

Table 7.1. *Experimental conditions for ICP-MS analysis*

Parameter	Setting
Rf power	1.20 Kw
Plasma gas flow rate	8.0 L/min
Carrier gas flow rate	0.70 L/min
Auxiliary gas flow rate	1.10 L/min
Chamber Temperature	5 ° C
Sampling Depth	5.0 mm
Plasma torch	Mini-torch
Spray Chamber type	Electronically-cooled cyclonic chamber
Nebulizer type	Coaxial
Monitored Signal	*m/z 75* (^{75}As), *m/z 111* (^{111}Cd), *m/z 112* (^{112}Cd), *m/z 114* (^{114}Cd), *m/z 59* (^{59}Co), *m/z 52* (^{52}Cr), *m/z 53* (^{53}Cr), *m/z 63* (^{63}Cu), *m/z 65* (^{65}Cu), *m/z 199* (^{199}Hg), *m/z 200* (^{200}Hg) , *m/z 201* (^{201}Hg), *m/z 202* (^{202}Hg), *m/z 55* (^{55}Mn), *m/z 58* (^{58}Ni), *m/z 60* (^{60} Ni), *m/z 207* (^{207}Pb), *m/z 208* (^{208}Pb), *m/z 78* (^{78}Se), *m/z 8* (^{88}Sr), *m/z 51* (^{51}V), *m/z 64* (^{64}Zn), *m/z 66* (^{66}Zn), *m/z 68* (^{68}Zn).

7.2.3.1. Validation of the ICP-MS method and metal quantification

The ICP-MS method was validated in terms of linearity, precision, accuracy, LOD and LOQ. Linearity was investigated in the range 0.1-30 μg L^{-1} through the building, performed automatically by the software, of calibration curves at eight concentration levels and five replicates for each level. Then, the quantification of metals in each unknown sample was performed automatically by the software. All the analyses, including the mineralization step, were performed in triplicates. Accuracy and precision were evaluated by the analysis of CRMs, mineralized three times and each digest sample was analyzed in triplicates. As for the accuracy, experimental quantitative results were compared with the values reported in the certificate of analysis, while the precision was estimated as inter-day repeatability through the analysis of CRMs in three different days, by calculating the coefficient of variation (CV%) of the measured concentrations. Instrumental and method LOD and LOQ were calculated by performing 10 replicates of the reagent blank and the sample blank (see section 7.2.2.1. "Sample preparation for metal analysis"), respectively. Then, LOD and LOQ were calculated using the following equations (Miller & Miller, 1993):

$$LOD = \frac{3\sigma C_{sp}}{I_{sp} - I_b} \qquad LOQ = \frac{10\sigma C_{sp}}{I_{sp} - I_b} \qquad [Eq.\,7.1]$$

where σ is the standard deviation of the signal intensity of 10 replicates, Csp is the final concentration of spiked metals (0.1 μg L^{-1} for all the metals in both reagent and sample blank), Isp and Ib are the signal intensity of the analyte in the spiked sample and a blank (nitric acid only), respectively.

7.2.3.2. Validation of the GC-MS method and Free Amino Acids quantification

FAA quantification was performed by external calibration with IS normalization in the range of 10-200 nmol mL^{-1} for all the standards (six concentration levels, five replicates for each level and only the points with a CV% minor than 20% were considered in the curve). In other words, calibration curves were built by plotting the ratio between the area of each peak and the area of the IS (y axis) against the standards concentration (x axis). The significance of the intercept was evaluated for each compound, and the curve was forced to zero when the P-value resulted higher than 0.05. The linearity of the curves was evaluated by the random distribution around zero of the calculated residuals, defined as the difference between the observed y value and the one predicted by the calibration curve for each x value. The precision of the method was estimated as repeatability (n=10) of the lowest point of calibration curve, by calculating the CV% of the ratio between the area of each peak and the area of the IS. LOD and LOQ were calculated, according to the Eurachem guidelines (Eurachem 2014), by performing 10 analyses of a reagent blank (20 mM HCl aqueous solution) spiked with a low concentration (10 or 20 nmol/mL) of analytes. The following equations were applied:

$$LOD = 3 \times s'0 \qquad LOQ = 10 \times s'0 \qquad [Eq.7.2]$$

where s'0 is the ratio among the standard deviation of the concentration measured through the calibration curves and the square root of the number of replicates.

7.3. Results and Discussion

7.3.1. Development and validation of the ICP-MS method

Besides the heavy and toxic metals mentioned in the introduction section for which strict regulation (Pb, Cd and Hg) exists or for which careful monitoring is highly recommended (As), other metals were included in this study due to their role in the human body and their widely reported presence in tuna fish. Moreover, a further selection was carried out based on the metals present in the CRMs available on the market for this application. The method here presented was successfully validated for Cu, Se, Cr, Co and Mn, metals considered essential to humans, and good results were also obtained for Ni, Sr and V for which neither toxicity nor essentiality has been definitely determined. For example, vanadium, a microelement present at ultra-trace amounts in both food and the human body, has been shown to have a variety of biochemical and physiological functions on the basis of its in vitro actions on cells and pharmacological actions in animals. However, in-vivo actions on humans have not been demonstrated yet. Its ability to mimic insulin by increasing the transport of glucose into the cell was one of the key functions for which it received particular attention. However, its role is pharmaceutical, rather than nutritional, as it is directed to a specific target population. As a result, it could be potentially toxic, and its daily intake should be minimum, as expected by a normal diet. The supplementation should occurs only under medical prescription [30]. On the other hand, strontium is a metal commonly present in nature, but only modestly absorbed by humans where it showed bone-seeking properties, similarly to calcium [31]. Finally, nichel compounds are classified in category I of carcinogenic substance, by the International Agency for Research for Cancer (IARC), indicanting that their carcinogenicity for human has been scientifically proven [56]. Genchi et al. [32] reported that a long-term exposure to foods containing high amounts of this metal promotes diseases such as emphysema, pulmonary fibrosis, and cancer of respiratory system. Table 7.2 shows the linearity range, instrumental and method LOD and LOQ for each metal. The linearity was investigated in the range 0.1-30 μg L^{-1}, but only the points with a coefficient of variation (CV%) less than 20% were used for quantification. The calibration curves, built daily immediately before the analysis of the unknown samples, always provided coefficient of linearity (R^2) higher than 0.9999 in the same linear range. The LOQ of the method was ≤ 0.1 mg Kg^1, for all metals investigated, indicanting that it can be used to monitoring total metal content in both fish muscles and fish oils (such as omega-3 supplements) in accordance with existing laws. The results of metal quantification in the CRM samples are presented in Table 7.3, in direct comparison to the values reported in the product certifications. The ratio between the measured and certified concentration was used to determine accuracy. The reliability of the quantification results in the unknown samples (see next section 8.3.2) ranged from 81% to 114% for all the metals in the three samples. Precision data was below 3% for all

the metals in a wide range of concentrations, specifically between 0.3 and 20 mg Kg^{-1}, with the exception of Hg in mussel tissue (ERM®-CE278k), where it was present at a level near to the method LOQ and measured with a precision of 14.5%. In this regard, the availability of three CRM allowed to ensure the precision and accuracy of the method at more concentration levels. Hg was present in all three samples in a concentration covering three order of magnitude, from 0.07 mg Kg^{-1} in the mussel tissue (ERM®-CE278k) to 0.6 mg Kg^{-1} in the fish muscle (ERM®-CE278k) up to 5 mg Kg^{-1} in tuna fish (ERM®-CE464).

Table 7.2. *Linearity range, Limits of Detection (LOD) and Limits of Quantification (LOQ) of the ICP-MS method for each analyzed metal.*

		Instrumental limits		Method limits	
Metal	**Linearity range (µg L^{-1})**	**LOD (µg L^{-1})**	**LOQ (µg L^{-1})**	**LOD (µg Kg^{-1})**	**LOQ (µg Kg^{-1})**
As	0.1-30	0.05	0.18	26.45	88.18
Cd	0.5-30	0.18	0.59	2.60	8.68
Co	0.5-30	0.21	0.70	32.66	108.86
Cr	0.5-30	0.22	0.74	29.47	98.24
Cu	1-30	0.23	0.77	9.86	32.85
Hg	2-30	0.48	1.61	20.88	69.60
Mn	1-30	0.28	0.95	13.79	45.96
Ni	1-30	0.25	0.83	13.00	43.33
Pb	1-30	0.24	0.81	16.18	53.95
Se	1-30	0.25	0.84	9.23	30.78
Sr	1-30	0.20	0.67	30.00	100.00
V	1-30	0.05	0.17	6.34	21.15

Table 7.3. *Certified and observed values (mg kg^{-1}) of heavy and essential metals in the CRM samples, along with accuracy and precision (CV%) data.*

Metals	Certified	Observed	Accuracy (%)	Precision (CV%, n=9)
FISH MUSCLE (ERM®-BB422)				
As	12.7 ± 0.7	12.0 ± 0.1	94.5	0.83
Cd	0.0075 ± 0.0018	< LOQ	-	-
Cu	1.67 ± 0.16	1.69 ± 0.04	101.2	2.37
Hg	0.601 ± 0.030	0.640 ± 0.010	104.9	1.56
Mn	0.368 ± 0.028	0.362 ± 0.004	98.4	1.10
Se	1.33 ± 0.13	1.13 ± 0.03	85.0	2.65
MUSSEL TISSUE (ERM®-CE278k)				
As	6.7 ± 0.4	6.15 ± 0.09	91.8	1.46
Cd	0.336 ± 0.025	0.300 ± 0.003	89.3	1.00
Cr	0.73 ± 0.22	0.73 ± 0.01	100.0	1.37
Cu	5.98 ± 0.28	6.11 ± 0.07	102.1	1.14
Hg	0.071 ± 0.007	0.069 ± 0.010	97.2	14.5
Mn	4.88 ± 0.24	4.51 ± 0.04	92.4	0.89
Ni	0.69 ± 0.15	0.600 ± 0.001	87.0	0.17
Pb	2.18 ± 0.18	2.17 ± 0.04	99.5	1.84
Se	1.62 ± 0.12	1.31 ± 0.01	81.0	0.76
Sr	19.0 ± 1.2	21.6 ± 0.3	114.0	1.39
TUNA FISH (ERM®-CE464)				
Hg	5.24 ± 0.10	5.41 ± 0.01	103.0	0.18

7.3.2. Quantification of metals

The results of metal quantification in the fresh tuna samples (edible fraction and waste) supplied by a local fish market in Messina (Sicily, Italy) are shown in Table 7.4. These samples, as well as the fractions obtained from the Bligh&Dyer extraction process, were used to make preliminary considerations about metal distribution between lipids and proteins, as well as to compare the metal concentration between edible (muscle) and waste products. Among hazardous and heavy metals, Hg and Ni were detected in these samples.

Table 7.4. *Metals quantified (mg kg^{-1}) in samples purchased in a local fish store in Messina (Sicily, Italy).*

Sample	As	Cr	Cu	Hg	Mn	Ni	Se
Muscle	2.30 ± 0.01	0.25 ± 0.00	0.64 ± 0.07	0.68 ± 0.01	< LOQ	< LOQ	0.40 ± 0.00
Waste	5.74 ± 0.08	0.76 ± 0.02	2.80 ± 0.08	0.45 ± 0.01	1.59 ± 0.02	0.35 ± 0.01	2.10 ± 0.04
Lipid Extract of the waste	7.83 ± 0.29	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
Protein layer of the waste	1.53 ± 0.01	0.13 ± 0.00	0.55 ± 0.05	1.53 ± 0.01	5.87 ± 0.04	0.36 ± 0.00	3.24 ± 0.11
Aqueous fraction of the waste	0.89 ± 0.02	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ

In both the muscle and the waste, mercury levels were below the legal limits. It was not detected in the lipid and aqueous fractions, but its concentration in the protein layer, where it was measured at level higher than 1 mg Kg^{-1}, suggesting that its quantify should be carefully monitored in case of the potential re-utilization of this fraction for the recovery of noble proteins or peptides. Similarly, Ni, which is only detected in the waste, is only found in the protein layer. As for the essential metals, all the investigated microelements were detected, with the exception of cobalt. They were discovered in higher concentrations in the wastes, and only protein layer contains considerable levels. In particular, Mn and Se were detected at levels of mg Kg^{-1}. Due to their role in the organism, their presence significantly increases the value of the waste in the context of the blue economy. Manganese is a cofactor of numerous enzymes involved in the metabolism of carbohydrates and AAs. Muscle weakness is caused by manganese deficiency [33]. Selenium is a constituent of selenoproteins, which are engaged in many oxidation/reduction reactions and protect cells from oxidative stress caused by reactive oxygen species (ROS) [34]. These proteins also catalyze the conversion of the thyroid hormone thyroxine into triiodothyronine, which is crucial for initiation and enhancement of immunity [35]. Finally, selenium has been shown to inhibit the accumulation of inorganic mercury [36]. The other metal found in all samples was As, measured at mg kg-1 levels. Unlike the other metals, following the Bligh&Dyer extraction, it was concentrated in the lipid extract, indicanting that tuna have a high content of arsenolipids; this is in accordance with the results of previous studies [37, 38] which identified some arsenolipids in different fish oils [39,40], fish products [40], and other marine organism such as algae [37]. Furthermore, high levels of As were detected in both the protein layer and the aqueous fraction, paving the way for more comprehensive speciation research, as recommended by EFSA authority [19]. The results on industry by-products and derived fractions were somewhat in agreement with these preliminary results.

Table 7.5 shows the results of metal quantification in the industrial tuna samples.

Table 7.5. *Metals quantified (mg kg^{-1}) in the industrial by-products of tuna.*

Sample	As	Cd	Co	Cr	Cu	Hg	Mn	Ni	Se	Sr	V
Raw waste	2.17 ± 0.02	2.92 ± 0.11	< LOQ	< LOQ	1.77 ± 0.11	0.17 ± 0.00	1.66 ± 0.01	0.35 ± 0.01	3.40 ± 0.13	48.9 ± 2.18	0.04 ± 0.00
Cooked waste	0.77 ± 0.02	0.04 ± 0.00	0.30 ± 0.02	0.34 ± 0.02	0.46 ± 0.05	0.28 ± 0.02	0.37 ± 0.01	0.34 ± 0.03	0.68 ± 0.05	14.7 ± 0.57	0.03 ± 0.00
Lipid fraction (raw waste)	1.24 ± 0.01	< LOQ	< LOQ	0.18 ± 0.01	< LOQ	0.10 ± 0.00	< LOQ	< LOQ	0.10 ± 0.01	< LOQ	< LOQ
Lipid fraction (cooked waste)	0.25 ± 0.01	0.02 ± 0.00	< LOQ	0.20 ± 0.01	< LOQ	0.10 ± 0.00	< LOQ	0.08 ± 0.01	< LOQ	< LOQ	0.04 ± 0.00
Aqueous fraction (raw waste)	1.94 ± 0.02	0.33 ± 0.00	< LOQ	< LOQ	0.46 ± 0.02	0.11 ± 0.00	< LOQ	0.09 ± 0.00	2.40 ± 0.08	0.90 ± 0.02	< LOQ
Aqueous fraction (cooked waste)	0.44 ± 0.01	< LOQ	< LOQ	< LOQ	0.07 ± 0.01	< LOQ	< LOQ	0.08 ± 0.00	0.22 ± 0.01	0.60 ± 0.01	< LOQ
Protein residue (raw waste)	3.80 ± 0.06	0.07 ± 0.00	< LOQ	< LOQ	0.08 ± 0.01	0.11 ± 0.00	< LOQ	0.20 ± 0.01	1.25 ± 0.06	0.78 ± 0.03	< LOQ
Protein residue (cooked waste)	1.80 ± 0.02	0.02 ± 0.00	< LOQ	< LOQ	0.18 ± 0.01	0.15 ± 0.01	< LOQ	0.25 ± 0.01	0.76 ± 0.04	1.34 ± 0.03	< LOQ

Mercury was quantified at levels of hundreds of mg Kg^{-1} in all the samples, below the limits set for tuna fish muscle, but equal or higher than the maximum concentration assigned for food supplements. As a result, in the context of the Blue Economy model, every step of the recycling process should be

closely monitored. Another toxic element, Cd, regulated to very rigorously low limits, was detected in the industrial waste. The raw waste contained an amount eight times higher than the legal limit, a slightly higher concentration in the aqueous fraction, indicating that this element is widely distributed in the water soluble fraction. Nichel was detected in both raw and cooked waste and has also been shown to be more concentrated in the protein phase after the separation process. High levels of As were detected in all the samples and derived fractions. However, differently from the liquid extraction procedure, the decantation process resulted in a significant accumulation of As in the solid protein residue. This confirms the need for speciation studies. With regard to the essential metals, Cu, Mn and Se were detected at levels of mg kg^{-1} in the raw waste, but only Cu and Se were further detected in the aqueous and protein residues, indicating that Mn is likely lost during the cooking process, possibly due to contacts with the employed metal surfaces. The presence of seleno-aminoacids, such as selenocystein and selenomethionine, in tuna fish can properly justify the high levels of Se in both the aqueous and protein residue [41]. Finally, with respect to the fresh tuna samples purchased in the local fish market, high levels of Sr, small amounts of the essential metal Co and trace levels of V were also detected. The levels of Sr are considerably reduced in the separated fractions, as a result of industrial processes.

7.3.3. Development and validation of the GC-MS analysis of Free Amino Acids

The chemical structures of the derivatized analytes was taken into account while choosing the columns. Alkyl chloroformate, in particular, reacts rapidly with both the amino and the carboxyl group of amino acids, converting them to amide and ester functional groups, resulting in volatile derivatives that can be analyzed with GC [42]. However, other functional groups present on AAs side chains, such as hydroxyl groups and amide groups, have relatively low reactivity [42], resulting in a wide range of polarity that makes GC separation difficult. Then, as medium-polarity column, a 50% phenyl polysiloxane stationary phase commonly used for the analysis of polycyclic aromatic hydrocarbons (SLB-PAHms, Merck Life Science) was chosen. Due to the high degree of functionalization, such a column provided better results for the separation of PAH isomers compared to a more conventional 5% phenyl polysiloxane phase [43,44]. In the present study, the column was heated to 320 °C, allowing the elution and the baseline separation of all the AA derivatives, covering the entire chromatographic space (Figure 7.1). In order to develop a fast GC method we used a custom version of the SLB-PAHms column not commercially available in the employed dimensions (10 m × 0.25 mm × 0.25 µm). A lab-constructed mass spectral library with embedded LRI, was used for compound identification. Implementing a dual-filter database allowed unambiguous differentiation between isomeric compounds, such as isoleucine and alloisoleucine, for which ion source fragmentation

produces nearly identical MS spectra, making it impossible to identify peaks without retention data. The AAs included in the database, viz. in this work, have been selected in order to cover all the main proteinogenic AAs. In particular, we inserted all the essential AAs (HIS, ILE, LEU, LYS, MET, PHE, THR, TRP and VAL) that cannot be synthesized by the organism and require to be introduced with the diet [45]. Moreover, some non-proteinogenic AAs, such as ABA, BAIB, TPR, AAA, APA and ORN, were also included for their interest in metabolomic researches [46-51]. Finally, we added histamine, a biogenic amine that functions mainly as a mediator of anaphylactic or allergic reactions. The method was validated in terms of LOD and LOQ, precision, and linearity. The results are reported in Table 7.6. In particular, LOD and LOQ values were low enough to allow the determination of all the compounds at trace level with high precision (CV% calculated at the lowest point of the calibration curve was ≤ 15.5% for all the compounds). As a first task, the method is suitable for the determination of histamine, according to the current legislation, and, as a second goal of the present research, a total of 28 FAAs can be quantified, as required prior to their use for nutraceutical formulations or for food supplements.

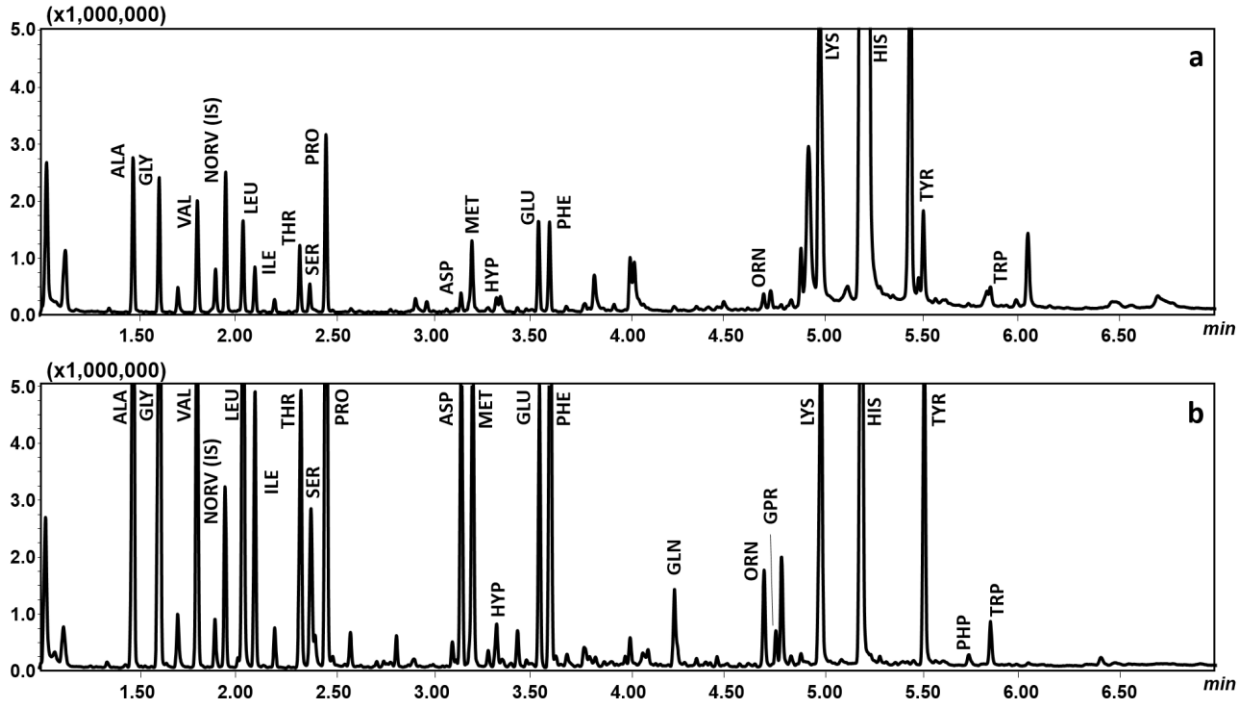

Figure 7.1. *GC-MS chromatograms of AAs derivatives in (a) tuna fish muscle and (b) waste*

Table 7.6. *Calibration curves parameters and LOD and LOQ values for each FAA derivative analyzed by GC-MS.*

FAA		LOD (nmol L^{-1})	LOQ (nmol L^{-1})	Linearity range (nmol L^{-1})	R^2	Precision (CV%) n=10	Slope	Intercept
Alanine	ALA	1.06	3.23	10-200	0.9979	3.11	0.0024	
Sarcosine	SAR	1.22	3.71	10-200	0.9953	3.78	0.0024	
Glycine	GLY	2.21	6.70	10-200	0.9948	6.72	0.0025	
α-Aminobutyric acid	ABA	2.18	6.61	10-200	0.9966	6.51	0.005	
Valine	VAL	2.48	7.50	10-200	0.9937	6.91	0.0041	0.0134
β-Aminoisobutyric acid	BAIB	2.66	8.05	10-200	0.9969	7.38	0.0031	0.0068
Leucine	LEU	2.00	6.07	10-200	0.9985	6.35	0.0051	
Alloisoleucine	AILE	1.87	5.66	10-200	0.995	5.01	0.0046	0.0179
Isoleucine	ILE	2.42	7.32	10-200	0.9918	6.16	0.0042	0.0237
Threonine	THR	2.08	6.29	10-200	0.9926	8.19	0.0038	
Serine	SER	1.84	5.59	10-200	0.9955	13.30	0.0014	-0.01
Proline	PRO	0.78	2.36	10-200	0.991	5.32	0.0052	
Asparagine	ASN	2.45	7.44	10-200	0.9937	9.50	0.003	
Thiaproline	TPR	3.48	10.53	10-200	0.9926	11.64	0.0042	
Aspartic acid	ASP	2.57	7.80	10-200	0.9928	7.63	0.0042	0.0159
Methionine	MET	2.21	6.70	10-200	0.9925	8.73	0.0067	
Proline, 4-hydroxy	HPR	2.67	8.09	10-200	0.9933	11.25	0.003	
Glutamic acid	GLU	2.26	6.86	10-200	0.9964	9.41	0.0028	-0.0057
Phenylalanine	PHE	1.86	4.72	10-200	0.9957	4.43	0.0082	0.0274
α-Aminoadipic acid	AAA	1.78	5.39	10-200	0.9975	7.35	0.0033	-0.0104
α-Aminopimelic acid	APA	2.71	8.22	20-200	0.9964	4.47	0.0049	
Glutamine	GLN	4.13	12.51	20-200	0.9916	12.76	0.0027	-0.0418
Histamine	HA	4.28	12.98	20-200	0.9838	11.06	0.0022	-0.0332
Ornithine	ORN	2.97	9.01	20-200	0.9969	8.68	0.0058	-0.0632
Proline, glycyl	GPR	2.07	6.27	20-200	0.9958	6.01	0.0069	-0.0646
Lysine	LYS	0.82	2.48	10-200	0.9972	10.63	0.006	-0.0641
Histidine	HIS	0.61	1.86	10-200	0.9905	15.48	0.004	-0.0607
Tyrosine	TYR	1.78	5.50	10-200	0.9971	7.88	0.0095	-0.0229
Tryptophan	TRP	1.18	3.57	10-200	0.9973	6.40	0.0091	-0.0371

7.3.4. Quantification of Free Amino Acids

The developed method has been used to determine the levels of FAAs in the edible fraction (muscle) as well as a mix of wastes obtained from a local fish market. The chromatograms obtained are reported in Figure 7.1, while the quantification of the identified FAAs is reported in Table 7.7.

Table 7.7. *Total amount (nmol mL^{-1}) of the identified FAAs in the edible fraction (muscle) and a mix of wastes acquired in a local fish store of Messina, Sicily.*

FAA	Waste	Muscle
Alanine	1407.85 ± 12.77	464.99 ± 4.86
Glycine	1175.38 ± 9.07	368.93 ± 6.09
Valine	600.69 ± 4.06	198.53 ± 3.61
Leucine	503.67 ± 4.63	128.15 ± 2.58
Isoleucine	306.87 ± 3.29	68.61 ± 1.88
Threonine	479.97 ± 13.91	116.91 ± 2.22
Serine	629.40 ± 19.84	133.46 ± 10.37
Proline	668.87 ± 8.58	240.17 ± 4.15
Thioproline	54.55 ± 2.12	-
Aspartic acid	259.26 ± 2.30	25.69 ± 1.57
Methionine	254.31 ± 3.11	76.39 ± 5.26
Proline, 4-hydroxy	76.01 ± 6.19	35.33 ± 2.93
Glutamic acid	665.82 ± 3.59	235.19 ± 5.83
Phenylalanine	280.96 ± 2.98	73.29 ± 1.06
Glutamine	407.70 ± 10.67	-
Ornithine	118.84 ± 3.42	28.82 ± 1.04
Proline, glycyl	36.18 ± 0.63	-
Lysine	285.33 ± 9.61	-
Histidine	884.29 ± 35.15	6353.90 ± 306.33
Tyrosine	145.25 ± 4.84	-
Proline-hydroxyproline	41.99 ± 1.73	-
Tryptophan	40.14 ± 1.46	76.86 ± 5.50

Histidine was the most abundant FAA in the muscle, which is in agreement with previous researches [6], making it highly susceptible to histamine formation, derived from HYS decarboxylation, and therefore to inducing scombroid syndrome upon consumption. On the other hand, the quantity of histidine in the waste mix was much lower, thus reducing the risk of food poisoning. With the exception of tryptophan, the levels of all the other FAAs were higher in the waste mix than in the muscle. Finally, it is important to note that the amount of almost all essential AAs was from 3 to 4 times higher in the waste, with LYS being detected only in this fraction. After that, the method was applied to the by-products obtained from an Italian fish industry. Table 7.8 shows the quantitative data for the FAAs detected in the aqueous fractions and in the protein layers derived from the raw and cooked wastes.

Table 7.8. *Total amount (nmol mL^{-1}) of the identified FAAs in the aqueous fractions and in the protein residueobtained from the raw and cooked waste provided by the fish processing industry.*

FAA	Raw Waste		Cooked Waste	
	Aqueous Fraction	Protein residue	Aqueous Fraction	Protein residue
Alanine	47407.09 ± 1482.94	22600.41 ± 431.18	2283.18 ± 125.98	1617.18 ± 209.90
Sarcosine	280.20 ± 7.58	-	85.08 ± 6.27	-
Glycine	11525.39 ± 689.39	6273.16 ± 366.87	878.97 ± 46.18	557.48 ± 29.41
α-Aminobutyric acid	297.43 ± 29.08	-	-	-
Valine	16338.10 ± 637.71	7137.55 ± 128.90	475.11 ± 31.30	553.97 ± 27.66
Leucine	33898.44 ± 734.89	12040.10 ± 138.12	928.35 ± 74.87	584.25 ± 34.41
Isoleucine	13539.37 ± 168.88	4336.04 ± 156.99	374.99 ± 20.65	253.70 ± 10.22
Threonine	11080.35 ± 562.90	4734.20 ± 186.48	436.67 ± 26.04	290.11 ± 19.38
Serine	15244.67 ± 908.59	7558.20 ± 604.23	785.71 ± 42.84	504.95 ± 52.82
Proline	13440.70 ± 294.55	5845.13 ± 158.74	528.51 ± 50.31	392.90 ± 46.45
Aspartic acid	20075.83 ± 354.64	8036.19 ± 470.41	203.00 ± 14.34	124.42 ± 8.71
Methionine	9812.61 ± 390.86	-	140.65 ± 13.43	-
Proline, 4-hydroxy	-	-	86.26 ± 8.82	-
Glutamic acid	19044.49 ± 549.50	11696.60 ± 456.92	1057.47 ± 55.43	1078.52 ± 79.47
Phenylalanine	16296.02 ± 369.80	5144.46 ± 29.50	216.71 ± 7.33	148.90 ± 3.17
Histamine	2167.11 ± 223.92	-	-	-
Ornithine	2975.14 ± 216.41	2705.75 ± 249.20	-	59.85 ± 7.49
Proline, glycyl	249.38 ± 18.50	-	-	-
Lysine	-	5771.13 ± 173.14	-	-
Histidine	14351.55 ± 548.72	6974.41 ± 615.74	41248.80 ± 2465.54	23047.78 ± 1529.38
Tyrosine	7000.61 ± 163.83	4603.59 ± 206.73	113.95 ± 8.36	116.29 ± 12.07
Tryptophan	2725.13 ± 198.50	820.15 ± 75.44	-	-

Both wastes were rich in FAAs, with a clear difference since the ones obtained from the cooked waste, being mainly composed of dark muscle, resembled the composition of the raw fish muscle previously analyzed with histidine as the major FAA, while the raw waste showed a concentration of 1 or 2 order of magnitude for the majority of the other FAAs. Furthermore, the presence of histamine in the aqueous fraction derived from the raw wastes is noteworthy, with a concentration of 2167.11 ± 223.92 nmol mL^{-1}, equivalent to 240.87 ± 24.89 mg kg^{-1}. This value is higher than EU's guideline of limit of 200 mg kg-1 for whole fish, but lowen then limit of 400 mg/kg for the fishery products [9]. Probably the raw wastes were not stored at temperatures ≤6 ° C allowing the conversion of free histidine into histamine by means of the endogenous bacteria. The cooking step used in order to obtain the lipid fraction inactivated the bacteria but had no effect on the already produced HA that was concentrated in the aqueous fraction. However, this fraction is rich in essential AAs, in particular leucine and isoleucine are about 36 times higher in the raw wastes aqueous fraction compared with the same fraction from cooked wastes. Moreover, other non-proteinogenic AAs, such as α-aminobutyric acid and ornithine, were detected and quantified mainly in the samples deriving from the raw waste. ABA

is a nonproteinogenic amino acid used for the synthesis of nonribosomal peptides such as ophthalmic acid. Irino et al. [46] demonstrated that α-aminobutyric acid protect against oxidative stress by modulating the homeostasis of glutathione and that ABA oral administration efficiently raises both circulating and myocardial glutathione levels and protects against induced cardiomyopathy in mice. ORN is a free amino acid that stimulated the production of growth hormone, which improve the metabolism of carbohydrates, proteins and lipids. Sugino et al. [51] demonstrated how oral ornithine administration promoted lipid metabolism, activated the urea cycle, and, in females, reduced the performance loss due to physical fatigue.

7.4. Conclusion

In the last years much effort has been put into the recovery and reutilization of industrial by-products, with particular interest in the activities related to the oceans, seas and coasts (Blue Economy). In this study, we developed and validated two analytical methods for the identification and quantification of metals and FAAs in the by-products of the tuna processing industry. Both methods were tested on wastes and muscle samples obtained from a local fish market and subsequently applied to industrial wastes obtained from an Italian fish company. The analysis of metals revealed the presence of essential elements such as selenium, copper, manganese in the raw wastes. On the other hand, some toxic metals, such as cadmium, mercury and nichel, were found not only in the raw wastes but also in some fractions obtained after industrial treatments such as cooking and mechanical separation procedures, thus driving the selection of the best method for a safe recycle. The FAAs analysis highlighted the presence of histamine, the biogenic amine responsible for the scombroyd syndrome, in the aqueous fraction deriving from the raw wastes composed mainly by head, tail and guts, probably due to bad storage. Nevertheless, the high concentration of essential amino acids and the presence of some non-proteinogenic amino acids such as α-aminobutyric acid and ornithine in this sample, could be of great interest for the production of high-added value products such as nutraceuticals and dietary supplements. The methods developed and validated in this research can be of potential interest for the analysis of byproducts deriving from various food processing industries, paving the way for the reutilization of such wastes as a primary resource in a new production cycle.

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[55] Commission Regulation (EC) No 1170/2009 of 30 November 2009 amending Directive 2002/46/EC of the European Parliament and of Council and Regulation (EC) No 1925/2006 of the European Parliament and of the Council as regards the lists of vitamin and minerals and their forms that can be added to foods, including food supplements.
[56] F. Rigano, A. Arigò, M. Oteri, R. La Tella, P. Dugo, L. Mondello, Chrom A J.1640 (2021) 461963.
[57] IARC (The International Agency for Research on Cancer). IARC Monogr. Eval. Carcinog. Risk Hum 100C (2012) 169–218.