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Manuscript: *"Comprehensive multidimensional liquid chromatography for advancing environmental and natural products research"* (Pedro F. Brandão, Armando C. Duarte, Regina M. B. O. Duarte*)

Highlights

> Progress and issues in 2D-LC for profiling complex organic matrices

> Best separation mechanisms in LC×LC for decoding the heterogeneity of complex samples

> Revisiting peak capacity and orthogonality in environmental & natural products research

> Hyphenated LC×LC in targeted/untargeted environmental & natural products research

Comprehensive multidimensional liquid chromatography for advancing environmental and natural products research

Pedro F. Brandão, Armando C. Duarte, Regina M. B. O. Duarte*

Department of Chemistry & CESAM, University of Aveiro, 3810-193 Aveiro, Portugal

* Corresponding Author: regina.duarte@ua.pt

Abstract

Chromatography is the separation method of choice in most laboratories worldwide. Nonetheless, the increasing need to decode complex samples has created a demand for better separation skills. The addition of extra separation dimensions to a conventional liquid chromatography system was one of the ways to answer to this demand. Although very common in proteomics and polymer research, the use of Multidimensional Liquid Chromatography (MDLC) coupled to high-resolution detectors for separation and analysis of environmental and natural products samples has yet to receive the deserved attention. This article presents a critical review on the most prominent of these comprehensive MDLC methods for targeted and untargeted analysis of complex environmental and natural products samples. This article also discusses the practical aspects of applying peak capacity and orthogonality concepts in MDLC analysis of complex matrices. It also addresses the limitations and challenges ahead for advancing environmental and natural products research using comprehensive MDLC.

Keywords

Multidimensional liquid chromatography; 2D-LC; LC×LC; Targeted and untargeted analysis; Complex samples profiling; Separation mechanisms; Natural organic matter; Wastewaters; Traditional Chinese medicines; High resolution detectors

1. Introduction

In laboratories worldwide, liquid chromatography (LC) methods, particularly those based on one-dimensional operation mode, are still the quintessential separation techniques. Depending on the detection method employed, significant information on the composition of analytes or eluting fractions can be achieved by using one-dimensional LC, 1D-LC (Figure 1). Even though these methods possess many advantages, they are still unable to fully resolve all the different co-eluting compounds in more complex matrixes, such as those of environmental and natural products samples. In order to solve this issue, alternative separation methods with higher peak capacity based on multidimensional liquid chromatography (MDLC) systems have been shaped to resolve as many compounds as possible. Of these, those based on two-dimensional liquid chromatography (2D-LC) have been widely applied, with direct impact on the overall separation (Figure 1). Nevertheless, there are no obvious limitations to increase the dimensionality for three or more dimensions, except for the successive increasing dilution of the sample. As depicted in Figure 1, and regardless of the employed separation method, the chemical information extracted from the analytical process is always dependent upon the detectors used.

<FIGURE 1 here>

Multidimensional chromatography may seem a modern advancement in separation technology; nonetheless, it was first described in 1944 [1]. More than three decades later, the first comprehensive two-dimensional liquid chromatography (LC×LC) method was reported by Erni and Frei [2]. When compared to conventional 1D-LC, the addition of an extra separation dimension poses additional complexity that lead to new challenges, including the selection of suitable chromatographic stationary phases (i.e., separation mechanisms), compatibility of separation modes, mobile phases, detection techniques (i.e., detection sensitivity), as well as optimization of separation conditions, and data analysis (i.e., new algorithms for processing 2D chromatograms). The extensive research using 2D-LC has also resulted in a range of different terminologies, which justified the need to suggest proper and unambiguous nomenclature and symbols to facilitate communication between analysts [3]. Indeed, since the seminal work of Erni and Frei [2], 2D-LC has emerged as a front-line tool for targeted analysis of samples of diverse complexity in a variety of areas. In this regard, readers interested in specific application areas are advised to consult the recent reviews, and references therein, on the use of 2D-LC in biopharmaceutical analysis and lipidomics [4-6], Traditional Chinese Medicines [7], food analysis [8], proteomics [9], and polymer analysis [10]. In most of the existing research and review works, there has been a huge effort to streamline the applicability of 2D-LC to efficiently and effectively resolve challenging complex samples. Recently, two excellent review works were published where a great deal of attention has been given to the fundamental principles [6] as well as technical progress, method development and optimization strategies [4], which are pivotal for the design of efficient 2D-LC separation approaches in the targeted analysis of complex samples. Although the present review may seem to overlap the review works of Stoll and Carr [6] and Pirok et al. [4] in a few topics (such as peak capacity and the use of 2D-LC in the analysis of Traditional Chinese

75 Medicine, later discussed), there are still important gaps and a few caveats when using
76 2D-LC, particularly LC×LC, for the comprehensive fingerprint of complex
77 environmental and natural products samples. Under this scenario, to advance the frontiers
78 of knowledge within this research field, it is mandatory to provide the scientific
79 community with an up-to-date and critical assessment on the use of 2D-LC strategies
80 coupled to high-resolution detectors for the separation, targeted and untargeted profiling
81 of complex environmental and natural products samples. This review builds upon those
82 earlier reviews and it aims to be an important guide for planning fit-for-purpose 2D-LC
83 strategies within this research field. Due to space limitations, this is not an exhaustive
84 review of previous studies using 2D-LC in environmental and natural products research,
85 but instead it provides the scientific community with a new perspective on the benefits of
86 using 2D-LC strategies for gaining new insights into the nature of those complex organic
87 matrices. The final section of this review addresses the challenges ahead to strengthen
88 and improve the current knowledge on the use of 2D-LC-based approaches online
89 coupled to high-resolution detectors to resolve the heterogeneity and thus advance
90 environmental and natural products research.
91
92 ***1.1. Heart-cutting and comprehensive 2D-LC strategies: setting up the scene***
93 It is of common knowledge to chromatographers in many application areas that there are
94 two main modes of operation in 2D-LC: heart-cutting and comprehensive [6]. Heart-
95 cutting 2D-LC, denoted in the literature as LC-LC, is a multidimensional methodology
96 comprising two or more chromatographic columns connected by a switching valve, which
97 ensures the selective and online transfer of specific fractions (e.g. a single peak, a specific
98 time segment, a portion of a peak) from one to another column. The LC-LC methodology
99 is especially suited for the separation of a limited number of target components since it

100 requires the definition of the collection time of the fractions, according to the elution
101 times of the components of interest. Thus, the main advantage of this method is that after
102 knowing the elution time of the components under study in the different columns, it
103 becomes possible to optimize a procedure sufficiently selective to separate a given
104 component independently of the complexity of the environmental matrix in which is
105 embedded. However, this advantage turns out to be its major limitation: when either the
106 components are unknown, or the standards required for the optimization process are not
107 available, then the application of this method becomes impracticable. Consequently, this
108 advantage/limitation makes this technique ideal for target analysis of a limited number of
109 compounds instead of a global characterization of the sample, regardless of the detection
110 system. This fact helps to understand the reason why this technique is associated to a low
111 degree of completeness of chemical information when compared to 1D-LC-based
112 techniques, as schematically shown in Figure 1. Besides, the selectivity of LC-LC limits
113 the overall information that can be obtained from other components in the sample. This
114 technique has been used in the last decades for the analysis of compounds present in
115 biological and environmental samples at low concentrations, such as trace-level
116 determination of low-molecular mass carbonyl compounds in air [11], determination of
117 acidic pesticides in soils [12], determination of endocrine disrupting compounds in water
118 [13] and determination of estrogens in sediments [14].
119 On the other hand, when the main objective is to carry out a non-targeted screening of a
120 given sample, comprehensive MDLC, namely LC×LC, is a more adequate option. In
121 order to attain a true comprehensive 2D separation, a few set of conditions must be
122 fulfilled; 1) the whole sample must be subjected to two independent separation
123 mechanisms within the same run; 2) the whole sample components passes through the
124 detection system or at least in equal percentages that guarantees that the obtained

chromatogram is representative of the entire sample; and 3) the resolution attained in the first dimension should be kept (as much as possible) in the second dimension [15-17]. These three main criteria were defined by Giddings and are also generally accepted for LC×LC [15-17].

Since some detectors provide information that can be considered as "multidimensional", as is the case of multichannel Mass Spectrometry (MS^n) or Diode Array (DAD) detectors, few researchers may consider the detection step as an additional dimension. Nonetheless, in MDLC, when one refers to "dimensions", usually it refers to "separation dimension" (e.g. separation columns). Although high degrees of orthogonality can be achieved in LC×LC by using suitable columns selectivities in the two dimensions (i.e., different retention mechanisms), the use of an additional dimension represented by the detection step may pose difficulties to retrieve useful information from the 2D chromatograms. Understandably, the use of an extra dimension leads to a more complex graphic representation of the acquired chromatographic data since these are typically represented as 2D contour plots or three-dimensional surfaces. This 2D chromatographic data typically contains a vast amount of information that needs to be further processed through different algorithms in order to be readily accessible to the analyst. In this field, three main approaches have been proposed to deal with LC×LC data structures [18]. In the first approach, data from the LC×LC is viewed as a set of consecutive one-dimensional chromatograms. These chromatograms are then treated individually, taking advantage of all the knowledge and large amount of software already available for data treatment in 1D chromatography. The second approach consists in dealing directly with the data matrix, which requires knowledge and expertise on complex chemometric algorithms. This approach is especially suited to deal with three or higher order data structures, such as data derived from 2D chromatographic systems coupled to multichannel detectors (e.g.

150 MS^n and DAD). Finally, the third approach, converts the 2D matrix data into an image
151 and uses the high diversity of image processing algorithms and tools for data handling
152 and treatment [18]. These different types treatment methods have been discussed in the
153 literature in detail and we suggest the works of Matos et al. [18] and Pierce et al. [19] for
154 the interested readers. It should also be mentioned that LC×LC coupled to high-resolution
155 detectors is an analytical tool much more entwined with qualitative rather than
156 quantitative analysis and, consequently, there are relatively few LC×LC studies in which
157 quantitative analysis is discussed [20]. Although the use of LC×LC systems seem to be
158 difficult by non-experts, the introduction of a commercial version of this equipment and
159 the development of more "user-friendly" data processing and treatment software, are very
160 likely to boost a growing interest for including these LC×LC techniques in laboratories
161 in order to deal with the complexity of environmental and natural products samples.
162 It is also important to note that LC×LC can be carried out either in online or offline modes.
163 In offline mode, sample is injected in a single conventional 1D-LC system, and fractions
164 of the effluent are manually collected and injected, at a later time, into a 1D-LC system
165 with a different separation column. As highlighted by Stoll and Carr [6], a couple of
166 interesting features can be assigned to offline LC×LC approach: (i) it does not require
167 very high speed separations in the second-dimension as in online LC×LC; (ii) the 2D
168 separation could be carried out using a single 1D-LC instrument; and (iii) high peak
169 capacities can be achieved, although at the cost of a high time of analysis. Nevertheless,
170 offline LC×LC is considerably more prone to sample contamination and losses than
171 online mode, which is of particular concern when dealing with complex samples, such as
172 those of environmental and natural products samples. Online LC×LC, on the other hand,
173 is much more technically challenging than its offline counterpart because it generally
174 requires the use of an automated switching system (e.g., a 6, 8 or 10-port valve), which

interfaces the first and second dimension columns, and collects fractions of first-dimension effluent and injects them into the second-dimension column. Indeed, most of the efforts and progress achieved in LC×LC research field, have been devoted towards the development of adequate interfaces (modulator) between the two separation dimensions. The modulation interface is really the center piece for a successful LC×LC separation, in parallel to the challenge of combining two solvent systems in order to prevent detrimental effects of first-dimension effluent into the second-dimension separation. We refer interested readers to Pirok et al. [4] work for further details on modulation and solvent compatibility issues. It is not surprising that automation offered by online LC×LC systems leads to more accurate, reproductive, repeatable 2D separations, being also less labor intensive than the offline mode. This is of particular interest for environmental and natural products research, since online LC×LC coupled to high-resolution multichannel detectors offers new opportunities to effectively and efficiently profile and map the entire sample, whose complexity is very difficult to address using 1D-LC or LC-LC.

2. Targeted *versus* untargeted analysis: finding the best separation conditions

LC×LC is adaptable to both targeted and untargeted analysis, but there are significantly different characteristics between both types of analysis. The concepts of peak capacity and orthogonality, that will be discussed later in more detail, are much more important in untargeted analysis than in targeted analysis. Targeted analysis aims at identifying some known compounds or confirming their presence in a sample. This type of analysis requires some pre-knowledge of the physicochemical properties of the compounds to be identified, thus meaning that it is confined to a relatively small number of well-studied

compounds. Since this type of analysis is very selective, and the compounds of interest are known, the chromatographic conditions can easily be optimized using standard solutions. Targeted analysis thus becomes very useful in environmental studies and attempts to find the best separation conditions is usually focused in increasing the sensitivity and selectivity of the analytical method to quantitively determine the analytes of interest. Untargeted analysis, on the other hand, is a non-selective search aiming at the identification of unknown components in a sample. In principle, this procedure is carried out without any *a priori* knowledge and information on the compounds to be identified. As highlighted by Matos et al. [21], it is impossible to achieve a complete untargeted analysis in chromatography, because all the choices made in terms of experimental conditions (e.g. the selection of stationary phases, the gradient and composition of mobile phases, and the detectors used) will be restricted to the scope of the analytical work as well as the range of properties associated to the compounds that can be separated and detected. Thus, finding the best separation conditions in untargeted analysis usually aims to increase the number of compounds that can be successfully detected and identified. Taking into account these constrains, the untargeted analysis can be further classified into two groups, depending on the analytical challenge or environmental problem to be solved: "fully untargeted" (now on referred just as untargeted), where there can be a vast number of unknown analytes present in the sample; and "semi-targeted", where some specific classes of compounds or some analytes are expected to be found [22]. Obviously, this classification between "fully untargeted" and "semi-targeted" must be kept in mind when developing new analytical LC×LC procedures to address a given problem.

Tables 1 and 2 summarize some important examples of LC×LC applications for the analysis of environmental and natural products samples from an untargeted and semi-targeted perspective, respectively. As shown in Table 1, the most common untargeted

LC×LC application is the profiling of natural products in plant extracts, particularly those used in Traditional Chinese Medicine (e.g. ginseng and other plants extracts). This is due to the complexity of these samples, encompassing hundreds or thousands of constituents with very different properties, and possibly with synergistic effects, where the quality control of these samples is a demanding issue [23]. When addressing the samples of interest from a semi-targeted point of view (in Table 2), LC×LC is commonly applied into the separation, with subsequent determination, of phenolic and polyphenolic compounds, also in natural products. Although there is great potential to apply LC×LC to environmental samples, this area has not developed that much at this point in time. Online LC×LC coupled to MS detector has been used for semi-targeted analysis of wastewater samples, allowing the identification of 23 to 65 compounds, including analgesics such as Paracetamol and Tramadol, herbicides Diuron and Monuron, Benzotriazole a known Corrosion inhibitor, and antidepressants such as Venlafaxine and Sertraline (references [24] and [25] in Table 2). LC×LC coupled to three detectors in series [UV, fluorescence detector (FLD), and evaporative light-scattering detector (ELSD)] was also applied to resolve the chemical heterogeneity of Suwannee River fulvic acid standard material and Pony Lake fulvic acid reference material (reference [26] in Table 2). Due to the complex nature of these samples, incompletely resolved fractions were still portrayed. Nevertheless, in cases where samples separation was accomplished (hydrophobicity × molecular weight), it was concluded that smaller molecular weight group fractions seem to be related to a more hydrophobic nature. Following this seminal work with complex natural organic matter, online LC×LC coupled to either a DAD and FLD in series [27], or a single DAD [21] was applied to exploit the compositional changes over a molecular size continuum and associated light-absorption properties of chemically distinct pools of urban organic air particles [i.e., water-soluble organic matter (WSOM)

and alkaline-soluble organic matter (ASOM)]. The results obtained in these two later studies highlight the potential of MLDC techniques, namely of online LC×LC coupled to high resolution detectors, for unravelling the complexity of the substructures present in complex environmental organic matrices. This fact constitutes a huge advantage in comparison to the traditional 1D-LC. Moreover, even if one tries to replicate these results using only 1D-LC techniques, this would need at least several chromatographic analysis and tedious procedures for collection of fractions.

<TABLE 1 here>

<TABLE 2 here>

In any chromatographic method, the optimization of the separation conditions is crucial, and LC×LC is no exception. Considerable efforts must be devoted in finding the best LC×LC separation conditions because there are many different factors that can significantly influence the final peak capacity. Selection of mobile phase composition in both dimensions and their respective compatibility and flow rates, the type of switching valve and the volume of the sampling loop, as well as selection of a fit-for-purpose detection system, are important factors to have in mind when developing a LC×LC-based method. Nonetheless, the most important factor when designing a LC×LC method is arguably the selection of the separation mechanisms (i.e., columns) to be employed in both dimensions, taking into account the analytical problem to be answered and whether the selected separation mechanisms are distinct from each other, but compatible at the same time. If successful in reaching this condition, enhanced orthogonality and peak capacity will be achieved. In this regard, the analytical problem to be solved plays in fact an important role in column selection. If dealing with a targeted analysis, the orthogonality is not a crucial outcome of the LC×LC procedure. The separation columns in both dimensions can share the same separation mechanisms, as long as they are

successful in answering the scientific question set *a priori*. In targeted analysis, there is only a few sets of compounds of interest, and a small difference between stationary phases can be enough to reach the desired separation. On the other hand, in semi-targeted and untargeted analysis, the scenario is completely different, and the train of thought must be necessarily different.

In the case of semi-targeted analysis, where the main purpose typically encompasses studying different sets of classes of compounds, it is important to take into account the structure of these analytes. For instance, if the compounds of interest all have low polarity, then it would be a wise choice to use a reversed-phase LC (RPLC) column in one of the dimensions, instead of a normal-phase LC (NPLC) column or even a hydrophilic interaction chromatography (HILIC) column (which can behave as a RPLC or NPLC, depending on mobile phase composition). Furthermore, the use of a RPLC×RPLC system can be suitable for the separation of some specific classes of compounds, such as phenolic compounds in Rooibos plants and Cocoa (references [28, 29], in Table 2), as long as the two stationary phases have different properties (e.g. different particle size, composition or different bonded phase). Other clear orthogonal combinations might be more difficult to accomplish, such as HILIC×SEC, SCX×RPLC, and NPLC×RPLC, due to possible mobile phase incompatibility. However, HILIC×SEC was already successfully used for resolving and profiling the chemical heterogeneity of natural organic matter from aquatic [26] and atmospheric matrices [21], whereas SCX×RPLC was employed for separation of low-molar-mass organic acids in different matrices [30] (Table 2). NPLC×RPLC is perhaps the least likely practical combination in terms of mobile phase compatibility, although exhibiting a high orthogonality from a theoretical point of view. As shown in Table 2, NPLC×RPLC has been applied for the separation of carotenoids [31, 32] (reported theoretical peak capacities of 651 and 986 [32]), and phenolic and stevioside

compounds [33] [reported peak capacities of 1850 (practical) and 3468 (theoretical)]. This NPLC×RPLC combination was only possible because the first-dimension encompassed columns either exhibiting characteristic features of HILIC systems (acting as NPLC) [33], or cyano microbore columns that offer normal-phase separations [31, 32]. On the other hand, if the desired type of analysis is untargeted, then using a RPLC×RPLC would probably not be enough to achieve the best orthogonality and peak capacity. However, the most common combinations in terms of separation mechanisms in natural products and environmental research is RPLC×RPLC, as shown in Table 1. In an untargeted analysis, it is questionable at first whether the combination of two or more RPLC columns, that would probably have similar separation characteristics, will yield a high orthogonality and peak capacity. Similar concerns are also valid when dealing with combination of any other stationary phases that separate compounds by polarity/hydrophobicity, such as HILIC, RPLC, and NPLC. Nonetheless, if aiming to achieve the maximum orthogonality with these sort of columns, then the separation conditions of the selected columns must be as much uncorrelated as possible. As shown in Table 1, this premise was successfully accomplished in the untargeted analysis of natural products [34-37] and household dust and laundry dryer lint [38] using RPLC×RPLC, where the reported orthogonality were as high as 93%. However, an interesting NPLC×RPLC combination is also noteworthy in the untargeted analysis of a traditional Chinese medicine [39] (Table 1). A silica column was chosen for NPLC in the first-dimension, and water-soluble non-polar 1,4-dioxane was selected as mobile phase modifier in NPLC. As a result, 876 peaks were detected, and the total peak capacity reached 1740 [39]. Since NPLC is suitable for group separation, and RPLC exhibits high resolution for less polar compounds, the potential of NPLC×RPLC combination for the analysis of complex environmental samples is enormous and should be further explored.

3. Updating and trends in peak capacity and orthogonality

The main point behind the use of two (or more) separation columns on a chromatographic system is to increase the maximum number of well resolved peaks, thus increasing the number of compounds that can be separated and further identified in a single chromatographic run. To accomplish this goal in LC×LC, a careful selection of the best combination of separations columns must be made. As discussed in Section 2, if the separation mechanisms in each chromatographic dimension are too similar, no significant advantage will be gained by using more than one dimension, mostly because the separation achieved with just one dimension will be basically the same, or lower in the following dimensions. In such situations, it may be better to use the conventional 1D-LC system, since there will be no loss of sensitivity caused by dilution of analytes in the following second or third chromatographic dimension. Accordingly, the ideal possible combination of columns should be those with completely uncorrelated separation mechanisms.

Full orthogonality is theoretically achieved if the separation mechanisms in all chromatographic dimensions are completely independent from each other [40]. In LC×LC, orthogonality varies between 0 and 100%, where 100% means that full orthogonality has been achieved [41]. In practice, however, it is very difficult to achieve full orthogonality in LC×LC, because this depends not only on the separation mechanisms in use, but also on the best separation conditions (e.g., mobile phase composition and flow rates) and samples characteristics [42]. In this regard, even the combination of two completely different separation mechanisms, such as SEC×RPLC, can show some correlation [42]. If we consider the concept of orthogonality in a more "visual" way, then a chromatogram where peaks are more disperse in the 2D space is considered to be more orthogonal compared to a situation where peaks are placed closer

350 to the diagonal of the 2D chromatogram (Figures 2A and 2F, respectively). It should be
351 also noted that orthogonality is a concept specific of each sample. Two distinct separation
352 columns that present high orthogonality for one given sample may not be adequate for
353 achieving an orthogonal separation of other samples. Therefore, chromatographic
354 separation conditions should be extensively studied in order to ensure a successful
355 separation outcome. Figure 2 illustrate possible consequences of a poor choice of column
356 combinations in LC×LC. Although exhibiting low correlation coefficient between the two
357 separation columns, second-dimension column in chromatograms B and C represents two
358 situations of an excessive and low interaction, respectively, between the analytes and the
359 stationary phase, which is not desirable for successful LC×LC separations. The same is
360 true in chromatograms D and E, where a poor choice of the first-dimension separation
361 column has a similar effect on the LC×LC separations.

362 <FIGURE 2 here>

363 When orthogonality is estimated, the value refers to the degree of separation of all
364 analytes in one specific sample. In fact, if samples are too different, it is difficult to carry
365 out a straightforward comparison of the orthogonality achieved. For example, if the
366 sample preparation step discards the polar compounds, then orthogonality obtained using
367 the common RPLC×RPLC system will be much higher than it would be if the polar
368 compounds had not been removed. The use of a standard mixture of dozens or even
369 hundreds of different compounds, with very distinct characteristics (e.g., molecular size,
370 polarity, structure) should be a more reliable way to estimate an "universal orthogonality"
371 for a given LC×LC system. The concept of peak capacity was defined by Giddings, in
372 1969, as "the upper limit of resolvable components for a given technique under prescribed
373 conditions" [43]. In LC×LC, it is generally accepted that the theoretical peak capacity is
374 simply the product of individual peak capacities in each dimension [17]. Obviously, the

theoretical peak capacity value represents the “best case scenario”. For example, it implies that no undersampling phenomena occurs and the resolution attained in one dimension is never lost in the subsequent dimension. Nonetheless, the process of sample transfer, from the first- to the second-dimension, will always lead to some resolution loss. Furthermore, even though peak capacity should be seen as a noteworthy way to measure the success of a separation process, we should also keep in mind that evenly spaced peaks in a 2D chromatogram are extremely unlikely to be found in any real samples. Interestingly, it has been shown that the number of well resolved peaks, in a given sample, is unlikely to be higher than 18% of total peak capacity of a LC×LC system [44]. As a consequence, the concepts of effective and practical peak capacity were developed as criteria to more accurately estimate the maximum number of peaks that can be effectively separated in a single 2D chromatographic run. The process of calculating the effective peak capacity is relatively simple. Briefly, a correction factor due to undersampling phenomena is applied to the theoretical peak capacity value, which will lead to a more realistic value for the effective peak capacity [45]. On the other hand, the concept of practical peak capacity is a bit more complex. As aforementioned, in LC×LC, it is almost impossible to achieve full orthogonality; therefore, by definition, in a 2D chromatogram there will exist always some areas that will never be occupied by any peak. This will obviously lead to a decrease in the available 2D chromatographic area where peaks can be separated, which will yield a practical peak capacity lower than the theoretical peak capacity [40].

The concepts of orthogonality and theoretical peak capacity, as well as those of practical and effective peak capacity, are important notions to have in mind when finding the best chromatographic conditions for implementing a LC×LC method. If the objective of studying new combinations of separation mechanisms in LC×LC is that they can be later

400 applied in profiling real complex samples, thus a good way these different LC×LC
401 methods can be compared is through those metrics. Obviously, these metrics should not
402 be the only criteria for comparing and optimizing different LC×LC methods;
403 nevertheless, the orthogonality and theoretical peak capacity should both be estimated in
404 any LC×LC method development. Unfortunately, these metrics are not often reported in
405 the literature [46], although in the last years some good examples of orthogonality and
406 theoretical, effective and practical peak capacity have been described, particularly in the
407 field of Chinese herbal medicine screening [35-37] (Table 1).
408
409
410 **4. Finding the best detection conditions**
411 In conventional 1D-LC, when a large number of analytes is present in a given sample,
412 their identification based on the comparison with a mixture of standard compounds is no
413 longer a feasible option. The coupling of chromatographic methods with a MS detector
414 is an excellent way to overcome this situation and attain the desired analytes
415 identification. Generally, studies summarized in Tables 1 and 2 employ as detection the
416 UV absorbance (either DAD or single UV wavelength), MS, or both detectors in series,
417 with the exception of two studies on natural organic matter that also use fluorescence and
418 evaporative light scattering detectors (Table 2) [26, 27]. Although a more comprehensive
419 identification of the analytes is overwhelmingly more frequent using MS, this does not
420 mean that the use of any other type of detector becomes pointless. It is possible to obtain
421 rather important information with the use of a DAD detector, since chromophores present
422 in a sample may have distinct absorption maxima that can be used to differentiate between
423 different molecules exhibiting similar *m/z* values in a MS detector [47].

Hence, in LC×LC, the use of a MS detector is rather common, and an enhanced separation before the MS detection has various advantages when compared to 1D-LC-MS. Some of these advantages include the reduction of matrix effects resulting from coeluting analytes due to the increased separation power of the LC×LC system, increased ionization efficiency and minimization of ion suppression [16]. On the other hand, the coupling of a MS detector to a LC×LC system is not as straightforward as in 1D-LC and some specific modifications have to be made. In LC×LC-MS, the mobile phase constituents, particularly in the second-dimension, must be volatile in order to be compatible with the MS source [16]. Thus, the second-dimension separation column must be compatible with mobile phases whose composition includes high percentage of organic solvents, such as methanol or acetonitrile, and volatile additives, such ammonium acetate and formic acid. In this regard, NPLC columns, or any other column that does not meet these requirements, can hardly be used as the last separation dimension before the MS detector. Notwithstanding this situation, the work developed by P. Dugo's research group is an excellent example of the use of NPLC in the first-dimension combined with RPLC in the second-dimension, and an MS as detector [31, 32, 48] [].

Another important requirement when coupling LC×LC to a MS detector is the sampling rate. It has been demonstrated by Murphy and co-workers [49], that the sampling rate of the first-dimension effluent has a significant influence on the resolution achieved in the second-dimension, suggesting that each peak in the first-dimension should be sampled at least three times. Therefore, the flow rate in the second-dimension is typically much higher than those used in the first-dimension. The extremely high flow rates in the second-dimension (up to 5 mL min^{-1}) are completely incompatible with any sort of MS detection. In the case of electrospray ionization (ESI), the maximum flow rate can be, at most, 1 mL min^{-1}, whereas in atmospheric-pressure chemical ionization (APCI) mode the maximum

449 flow rate is 2 mL min^{-1}. In practice, however, these flow rate values should be much lower
450 than the maximum values allowed [25, 50].The most common way to solve this issue in
451 LC×LC-MS is to use a flow splitter before the MS detector. This solution will allow the
452 detection of the analytes in the MS but will also greatly decrease the sensitivity of the
453 method. A more sophisticated response to this problem is the miniaturization of the entire
454 LC×LC system, which is more common in proteomics, but has also been successfully
455 employed by Haun and co-workers in wastewater analysis [25] (Table 2). The main
456 objective of this specific study was the construction of a miniaturized 2D-LC system,
457 coupled to a Quadrupole/Time-of-Flight Mass Spectrometric detection, for wastewater
458 profiling without the need to split the flow [25]. To decrease the time of analysis in the
459 second-dimension, high pressure and temperature conditions as well as a stationary phase
460 of superficially porous sub-3-μm were employed. The miniaturization of the LC×LC
461 system leads also to much lower solvent consumption. Using a standard mixture of 99
462 target compounds, the miniaturized LC×LC-MS system allowed the detection and
463 identification of 65 standard compounds in the wastewater samples. Despite the obvious
464 advantages over other LC×LC-MS systems, the work of Haun et al. [25] exhibits a real
465 possibility of an excessive decrease in system sensitivity when compared to a 1D-LC-MS
466 system, due to the dilution effect in the second-dimension separation. It should also be
467 taken into account that MS identification does not strictly require a complete
468 chromatographic separation of the analytes. To assess these points, another work, using
469 the same miniaturized LC×LC-MS system, was later published, with the objective of
470 comparing its sensitivity to that of a conventional 1D-LC-MS system in the analysis of
471 wastewater samples [24]. It was reported that the absolute intensity of the signal in the
472 LC×LC-MS system was ten times lower of that of the 1D-LC-MS system. However, this
473 difference only led to a small decrease in sensitivity because the signal-to-noise ratio was

474 only around 1.5 times lower when using the miniaturized LC×LC-MS. It was also
475 reported that the number of identified compounds was always higher in the miniaturized
476 LC×LC-MS system. Nevertheless, the main problem with miniaturization of the LC×LC-
477 MS system is probably its cost due to the use of expensive nanoLC pumps. However, the
478 simple decrease of the mobile phase flow rate in the second-dimension, to the range of
479 1-2 mL min^{-1}, should have beneficial effects. Even if this means that a flow splitter is still
480 necessary, at least the second-dimension effluent does not have to be split as much as in
481 other LC×LC-MS systems, which will lead to better results in terms of sensitivity.
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484 **5. Conclusions**
485 Over the past 10-15 years, the emergence of more effective systems and analytical
486 methodologies based on online LC×LC separations has become a clear trend in natural
487 products research. Surprisingly, and despite the advent of LC×LC-based methods, the use
488 of LC×LC methods is yet to be fully exploited in environmental research. The importance
489 of these methods is not only associated with the separation of the sample components, but
490 also with the rapidly evolving field of analytical instrumentation which has produced
491 more sophisticated detectors capable of providing a higher discrimination power. It is
492 clear from a vast assortment of studies in the literature that the use of hyphenated 1D-LC
493 methods (e.g. LC–MS and LC–NMR, Figure 1) has been able to provide new insights on
494 the compositional features of highly complex samples, a know-how which was
495 unforeseeable not long ago. However, the continuous development and use of MDLC,
496 namely online LC×LC, has shown the potential to provide deeper and more complete
497 knowledge into the structural complexity of environmental and natural products samples,

498 despite several technological challenges that still needs to be overcome to attain its full
499 capability.
500 Most environmental and natural products studies using LC×LC-MS as analytical
501 technique have focused either on screening or identification of a small sets of compounds
502 (i.e., in untargeted and semi-targeted analysis, respectively). The use of LC×LC in
503 environmental research has also focused on the global characterization of samples for
504 achieving a heuristic understanding of the complex structural nature and
505 interrelationships between different components within the samples. Therefore, it is with
506 no surprise that major improvements in the stationary phase technology in both LC
507 dimensions aiming at reaching orthogonality are still required, namely for acquiring a
508 better understanding of the interactions that occur between the samples' components and
509 the stationary phase. As a further step, it is necessary to reduce or even eliminate the
510 confounding effects due to these interactions occurring in the chromatographic analysis.
511 This step will be particularly challenging, but it will be mandatory for the identification
512 of the interactions that really occur within the environmental and natural products
513 samples. Without a clear idea of the mechanisms that occur in the separation process, it
514 will never be possible to assess the complexity of an environmental or natural product
515 sample, simply because the decoding of the complexity of such samples cannot be
516 accomplished following a separation process equally complex. Nevertheless, if able to
517 solve these methodological challenges, LC×LC-based methods can be a promising tool
518 for advancing environmental research and achieve a deeper level of knowledge within
519 this field.
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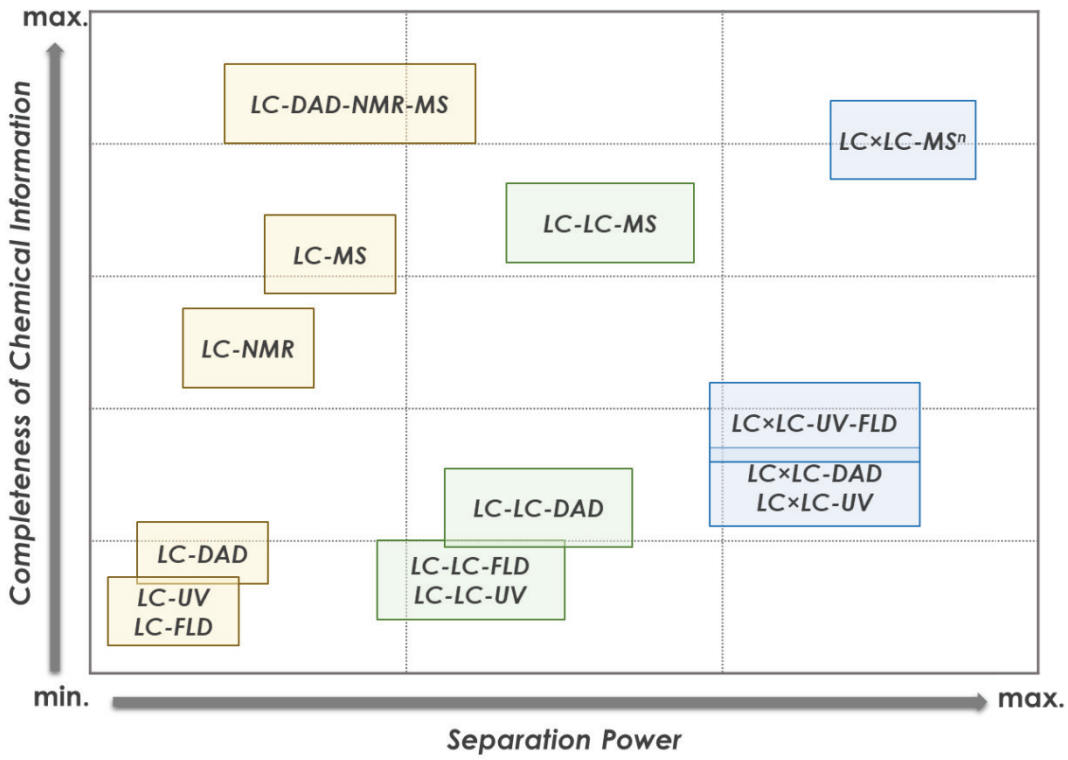
FIGURES CAPTIONS

Figure 1. Schematic representation of the range of LC-based techniques currently employed in environmental and natural products research as a function of their separation power and completeness of chemical information achieved. Acronyms: LC – one-dimensional liquid chromatography; LC-LC – two-dimensional heart-cutting LC; LC×LC – two-dimensional comprehensive LC; UV – ultraviolet detector; FLD – fluorescence detector; DAD – diode array detector; MS^n – mass spectrometry detector; NMR – nuclear magnetic resonance spectroscopy.

Figure 2. Schematic representation of LC×LC separations, exhibiting different levels of orthogonality (A, B, C, D, and E) and very low orthogonality (F). ^1D = first-dimension, ^2D = second-dimension.



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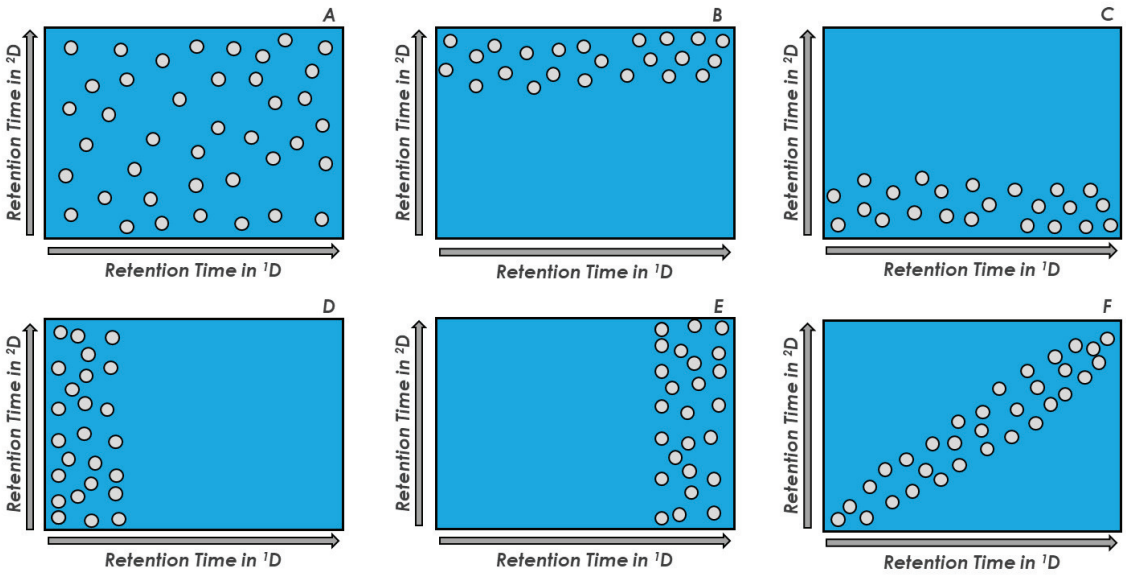

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765 **FIGURE 2**

766 **Table 1.** Examples of LC×LC applications for untargeted analysis of environmental and natural products samples. RPLC = Reversed-phase liquid
767 chromatography column, NPLC = Normal-phase liquid chromatography column, ILLC = Immobilized liposome chromatography
768 column, WAX = Weak anion-exchange chromatography column, MS = Mass Spectrometry, ^{1}D = first-dimension, ^{2}D = second-
769 dimension, TCM = Traditional Chinese Medicine.

Sample	Analytes	Column Set	Detection	Observations	Reference
Zhengtian pills (TCM)	876 compounds	NPLC×RPLC (2 tandem columns in ^{2}D)	UV (240 nm)	**Extraction Method:** Soxhlet with Ethanol; **Mobile phase:** ^{1}D: n-Hexane/1,4 dioxane (99.5:0.5), 100 µL/min; ^{2}D: Isopropanol/H_2O (2:98) and Methanol, 1 mL/min; **Theoretical Peak Capacity:** 1740	[39]
Magnolia-vine *(Schisandra chinensis)*	More than 40 compounds detected, 14 identified	ILLC×RPLC	UV (254 nm) and MS	**Extraction Method:** Ultrasound assisted extraction with n-Hexane; **Mobile phase:** ^{1}D:10mM Ammonium Acetate Solution at 1 mL/min; ^{2}D: H_2O and Acetonitrile at 3 mL/min;	[51]
Red Wine	Sample profiling	WAX×RPLC	DAD	**Mobile phase:** ^{1}D: 50 mM Phosphate buffer with 25% Methanol, 50 µL/min; ^{2}D: 50 mM Phosphate buffer with 25% Methanol, 3 mL/min;	[30]
Roots of *Pueraria lobata* and *Pueraria thomsonii*	Sample profiling	RPLC×RPLC	DAD and MS	**Extraction Method:** Ultrasound assisted extraction with Methanol; **Mobile phase:** ^{1}D: Methanol and Formic Acid 0.1% at 100µL/min; ^{2}D: Acetonitrile and Formic Acid 0.1% at 2.5 mL/min; **Theoretical Peak Capacity:** 3245 **Effective Peak Capacity:** 677 **Practical Peak Capacity**: 1593 **Orthogonality:** 68.5% **Additional information:** heart-cutting method was also used for sample characterization.	[36]
Sewage treatment plant effluents	20 compounds	RPLC×RPLC	UV (290 nm) and MS	**Extraction Method:** Soxhlet with Acetone and Methanol; **Mobile phase:** ^{1}D: H_2O and Acetonitrile; 100 µL/min; ^{2}D: Formic Acid 0.1% and Acetonitrile with 0.1% of Formic Acid at 2 mL/min;	[52]

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771 **Table 2.** *Cont.*

Sample	Analytes	Column Set	Detection	Observations	Reference
Household dust and laundry dryer lint	Plasticizers, flame retardants, pesticides, drug metabolites	RPLC×RPLC	MS	**Extraction Method:** Ultrasound assisted extraction with Methanol; **Mobile phase:** ^{1}D: H_2O and Acetonitrile at 100 µL/min; ^{2}D: Formic Acid 0.1% and Acetonitrile with 0.1% Formic Acid at 2 mL/min; **Orthogonality:** 67 (Household dust) and 73% (Laundry dryer lint)	[38]
Chinese medicine formula	280 compounds	RPLC×RPLC	UV (270 nm) and MS	**Extraction Method:** Ultrasound assisted extraction with Methanol; **Mobile phase:** ^{1}D: Acetonitrile and Formic Acid 0.1% at 100 µL/min; ^{2}D: Solution of Ammonium Hydroxide 10% and Ammonium Acetate 10mM at 2 mL/min; **Theoretical Peak Capacity:** 2763 **Effective Peak Capacity:** 710.3 **Practical Peak Capacity**: 1628 **Orthogonality:** 84.1%	[37]
Dengzhan Shengmai (TCM)	283 compounds (phenolic acids, flavonoids, saponins and lignan)	RPLC×RPLC	UV (280 nm) and MS	**Extraction Method:** Ultrasound assisted extraction with Ethanol; **Mobile phase:** ^{1}D: Formic Acid 0.1% and Methanol with 0.1% of Formic Acid at 100 µL/min; ^{2}D: Formic Acid 0.1% and Acetonitrile with 0.1% Formic Acid at 2 mL/min; **Effective Peak Capacity:** 1123 **Correlation Coefficient of the two columns:** 0.414	[34]
Curcuma *(Curcuma kwangsiensis)*	105 compounds	RPLC×RPLC	DAD and MS	**Extraction Method:** Solid-liquid extraction with Methanol; **Mobile phase:** ^{1}D: Acetonitrile and Formic Acid 0.1% at 20 µL/min; ^{2}D: Acetonitrile and Formic Acid 0.1% at 0.7 and 2 mL/min; **Theoretical Peak Capacity:** 1825 **Effective Peak Capacity:** 430 **Practical Peak Capacity:** 1416 **Orthogonality:** 93.2%	[35]

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774 **Table 3.** *Cont.*

Sample	Analytes	Column Set	Detection	Observations	Reference
Liquorice *(Glycyrrhiza glabra)*	120 compounds were detected 37 were identified	RPLC×RPLC	DAD and MS	**Extraction Method:** Ultrasound assisted extraction with Ethanol and H_2O; **Mobile phase:** ^{1}D: Acetic Acid 0.1% and Acetonitrile with 0.1% Acetic Acid at 10 µL/min;^{2}D: Acetic Acid 0.1% and Acetonitrile with 0.1% Acetic Acid, flow not specified; **Additional Information:** Multi-segmented shift gradients (MSG) and Full in-fraction modes were compared (FIF). **Theoretical Peak Capacity:** 1219 (MSG) and 654 (FIF) **Practical Peak Capacity:** 695 (MSG) and 260 (FIF) **Orthogonality:** 92 (MSG) and 50% (FIF)	[53]
Extracts of Hop cones and pellets (*Humulus lupulus*)	83 compounds were identified using the 2D-LC method	RPLC×RPLC	DAD and MS	**Extraction Method:** Solid-liquid extraction with n-Hexane; **Mobile phase:** ^{1}D: 10 mM Ammonium Acetate (pH adjusted to 9.0 with Ammonium Hydroxide) and Acetonitrile at 30 µL/min; ^{2}D: Acetic Acid 0.1% and Acetonitrile with 0.15 Acetic Acid at 2.2 mL/min; **Theoretical Peak Capacity:** 2418 **Effective Peak Capacity:** 756 **Practical Peak Capacity:** 1478	[54]

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Table 2. Examples of LC×LC applications for semi-targeted analysis of environmental and natural products samples. RPLC = Reversed-phase liquid chromatography column, NPLC = Normal-phase liquid chromatography column, HILIC = Hydrophilic interaction chromatography column, SEC = size-exclusion chromatography column, SCX = strong cation-exchange chromatography column, MS = Mass Spectrometry, FLD = Fluorescence detector; ELSD = Evaporative light scattering detector.

Sample	Analytes	Column Set	Detection	Observations	Reference
White and red ginsengs	Ginsenoids	HILIC×RPLC	MS	**Extraction Method:** Ultrasound assisted extraction with Methanol; **Mobile phase:** ^{1}D: 10 mM Ammonium Formate with 0.2% Formic Acid and Acetonitrile with 0.2% Formic Acid at 150 µL/min; ^{2}D: Formic Acid 0.2% and Acetonitrile, 0.6 mL/min; **Additional Information:** Other RPLC column was used as trap column. **Effective Peak Capacity:** 4392 **Orthogonality:** 55%	[55]
Animal Urine	Sulphonamides, β-agonists and Hormones	RPLC×RPLC	DAD and MS	**Mobile phase:** ^{1}D: H_2O/Acetonitrile (90:10) with 0.1% Formic Acid and H_2O/Acetonitrile (10:90) with 0.1% Formic Acid, 40 or 60 µL/min; ^{2}D: H_2O/Acetonitrile (90:10) with 0.1% Formic Acid and H_2O/Acetonitrile (10:90) with 0.1% Formic Acid, 0.8 or 0.35 mL/min; **Limits of Detection:** 1-10 µg/L	[56]
Chestnut tannin extract, Red Wine, Grape seeds and Rooibos tea	Phenols	HILIC×RPLC	DAD and MS	**Mobile phase:** ^{1}D: Formic Acid 0.1% and Acetonitrile, 9 or 11 µL/min; ^{2}D: Formic Acid 0.1% and Acetonitrile, 2.6 or 3 mL/min;	[57]
Cocoa	Phenolic Compounds	HILIC×RPLC	DAD	**Mobile phase:** ^{1}D: Acetonitrile with 1% Acetic Acid and methanol/H_2O/Acetic Acid (94.05:4.95:1), 4-40 µL/min; ^{2}D: Formic Acid 0.1% and Acetonitrile with 0.1% Formic Acid, 1-5 mL/min; **Practical Peak Capacity:** 1417-2430	[29]

787 **Table 2.** *Cont.*

Sample	Analytes	Column Set	Detection	Observations	Reference
Grapes, Grape seeds, rooibos tea and wine	Flavonoid and non-flavonoid phenolic classes	HILIC×RPLC	DAD and MS	**Extraction Method:** Solid-liquid extraction with different solvents; **Mobile phase:** ^{1}D: Acetonitrile with 0.1% Formic Acid and Formic Acid 0.1%, 11 µL/min; ^{2}D: Formic Acid 0.1% and Acetonitrile, 2.6 or 3 mL/min; **Practical Peak Capacity:** 2000-2600 (depending on sample) **Orthogonality:** 50-71% (depending on sample)	[58]
Vitis vinifera L. canes	Bioactive (poly)phenolic compounds	HILIC×RPLC	DAD and MS	**Extraction Method:** Ultrasound assisted extraction with Ethanol and H_2O; **Additional Information:** three and two different separation columns were tested on ^{1}D and ^{2}D respectively. **Mobile phase:** ^{1}D: i) Acetonitrile and 10 mM Ammonium Acetate, 15 µL/min; ii) Formic Acid 0.1% and Methanol with Formic Acid 0.1%, 20 µ/min; iii) Acetonitrile with 1% Formic Acid and Methanol/10 mM ammonium Acetate/Acetic Acid (95:4:1), 18 µL/min; ^{2}D: i) Formic Acid 0.1% and Acetonitrile with 0.5% Formic Acid, 3 mL/min; ii) Formic Acid 0.1% and Acetonitrile with 0.5% Formic Acid, 3 mL/min; **Theoretical Peak Capacity**: 943, 1188 and 1408; **Effective Peak Capacity:** 538, 432 and 842; **Practical Peak Capacity:** 768, 961 and 1080 **Orthogonality:**70, 45 and 78%	[59]
Apiaceous Vegetables	Furanocoumarins	RPLC×RPLC	DAD	**Extraction Method:** Solid-liquid extraction with H_2O and Acetonitrile with 0.1% Acetic Acid followed by QuEChERS; **Mobile phase:** ^{1}D: 5 mF Sodium Phosphate and Methanol, 250 µL/min; ^{2}D: 20 mM Phosphoric Acid and Acetonitrile, 2.5 mL/min;	[60]

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790 **Table 2.** *Cont.*

Sample	Analytes	Column Set	Detection	Observations	Reference
Italian apple cultivar	Multiple polyphenolic classes	HILIC×RPLC	DAD and MS	**Mobile phase:** [1]D: H_2O/Acetonitrile (80:20) with 0.1% Acetic Acid and Acetonitrile with 0.1% Acetic Acid, 100 µL/min; [2]D: Acetic Acid 0.1% and Acetonitrile with 0.1% Acetic Acid, 2.2 mL/min; **Theoretical Peak Capacity:** 1434, 1529 and 1946 **Practical Peak Capacity:** 867, 925 and 1180	[61]
Rubus idaeus shoots	Phenolic Compounds	RPLC×RPLC	DAD and MS	**Extraction Method:** Soxhlet extraction using Methanol and Chloroform; **Mobile phase:** [1]D: H_2O/Acetonitrile (50:50) with 0.1% Trifluoroacetic acid, 100 µL/min; [2]D: Trifluoroacetic acid 0.1% and H_2O/Acetonitrile (50:50) with 0.1% Trifluoroacetic acid, 1 mL/min; **Theoretical Peak Capacity:** 173	[62]
Pouteria sapote	Carotenoids	NPLC×RPLC	DAD and MS	**Extraction Method:** Solid-liquid extraction with Acetone; **Mobile phase:** [1]D: n-Hexane, Butyl acetate and Acetone (80:15:5) and n-Hexane, 10 µL/min; [2]D: 2-propanol and Acetonitrile with 10% H_2O, 3 mL/min;	[31]
Urban organic aerosols	Water-Soluble Organic Matter and Alkaline-Soluble Organic Matter	HILIC×SEC	UV (254 nm) and FLD (λExc: 240 nm/ λEm: 410 nm)	**Extraction Method:** Extraction from filters with H_2O; **Mobile phase:** [1]D: 20 mM of Ammonium Acetate (pH adjusted to 6.0 with acetic acid) with 10% of Acetonitrile, 20 or 17 µL/min; [2]D: 20 mM Ammonium Hydrogen Carbonate with 11% of Acetonitrile, 2.5 mL/min;	[27]
Black chokeberry pomace (*Aronia melanocarpa*)	Anthocyanins, proanthocyanidins, flavonoids and phenolic acids	HILIC×RPLC	DAD and MS	**Extraction Method:** Pressurized Liquid Extraction with H_2O, Formic Acid and Ethanol; **Mobile phase:** [1]D: Acetonitrile with 1% Formic Acid and 5% Formic Acid, 18 µL/min; [2]D: Formic Acid 0.1% and Acetonitrile, 3 mL/min; **Practical Peak Capacity:** 1287 **Orthogonality:** 76%	[63]

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793 **Table 2.** *Cont.*

Sample	Analytes	Column Set	Detection	Observations	Reference
Sargassum muticum	Phlorotannins	HILIC×RPLC	DAD and MS	**Extraction Method:** Pressurized Liquid Extraction with Ethanol and H_2O; **Mobile phase:** ^{1}D: Acetonitrile with 2% Acetic acid and Methanol/H_2O/Acetic Acid (95:3:2), 15 µL/min;^{2}D: Formic Acid 0,1% and Acetonitrile, 3 mL/min; **Theoretical Peak Capacity:** 1050 and 906	[64]
Natural organic matter (NOM)	Suwannee River and Pony Lake Fulvic Acids	HILIC×SEC	UV (254 nm) FLD (λExc: 240 nm/ λEm: 450 nm) and ELSD	**Mobile phase:** ^{1}D: 20 mM of Ammonium Acetate (pH adjusted to 6.0 with acetic acid) with 10 or 20% of Acetonitrile, 20 µL/min; ^{2}D: 20 mM Ammonium Hydrogen Carbonate with 11% of Acetonitrile, 2 mL/min;	[26]
Atmospheric Aerosols	Water-Soluble Organic Matter	HILIC×SEC	DAD	**Extraction Method:** Extraction from filters with H_2O; **Mobile phase:** ^{1}D: 20 mM of Ammonium Acetate (pH adjusted to 6.0 with acetic acid) with 10% of Acetonitrile, 20 µL/min; ^{2}D: 20 mM Ammonium Hydrogen Carbonate with 11% of Acetonitrile, 2.5 mL/min;	[21]
Wastewater	23 target compounds	RPLC×RPLC	MS	**Mobile phase:** ^{1}D: Formic Acid 0.1% and Methanol, 0.2 µL/min; ^{2}D: Formic Acid 0.1% and Acetonitrile, 0.040 mL/min;	[24]
Red Wine	Anthocyanins and derived pigments	HILIC×RPLC	UV (500 nm) and MS	**Mobile phase:** ^{1}D: Trifluoroacetic acid 0.4% and Acetonitrile with 0.4% Trifluoroacetic acid, 1 µL/min; ^{2}D: Formic Acid 7.5% and Acetonitrile with 7.5% Formic Acid, 0.86 mL/min; **Theoretical Peak Capacity:** 1386 **Practical Peak Capacity:** 889	[65]
Wastewater	65 target compounds	RPLC×RPLC	MS	**Mobile phase:** ^{1}D: Formic Acid 0.1% and Methanol, 0.2 µL/min; ^{2}D: Formic Acid 0.1% and Acetonitrile, 0.040 mL/min; **Orthogonality:** 61%	[25]

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796 **Table 2.** *Cont.*

Sample	Analytes	Column Set	Detection	Observations	Reference
Rooibos (Aspalathus linearis)	Phenolic Compounds	HILIC×RPLC	DAD and MS	**Extraction Method:** Solid-liquid extraction with H_2O; **Mobile phase:** [1]D: Acetonitrile with 2% Acetic Acid and Methanol/H_2O/Acetic Acid (93.05:4.95:2.00), 25 µL/min; [2]D: Acetic Acid 1% and Acetonitrile, 25 µL/min, 1.2 mL/min; **Theoretical Peak Capacity:** 830 **Practical Peak Capacity:** 415 **Correlation Coefficient of the two columns:** 0.659	[28]
Citrus juices	Flavones	RPLC×RPLC	DAD and MS	**Mobile phase:** [1]D: Formic Acid 0,1% and H_2O/Acetonitrile/Isopropanol/Formic Acid; (39.9:20:40:0.1), 50 µL/min; [2]D: Formic Acid 0,1% and H_2O/Acetonitrile/Isopropanol/Formic Acid (39.9:20:40:0.1), 3 mL/min; **Additional Information:** Limits of Detection and Quantification were calculated for Hesperidin (0.432 and 0.688 µg/mL) and Naringin (0.302 and 0.482 µg/mL)	[66]
Stevia rebaudiana	Polyphenolic and stevioside compounds	NPLC×RPLC	DAD	**Extraction Method:** Vortex assisted extraction with Acetonitrile and H_2O; **Mobile phase:** [1]D: Phosphoric Acid 0.004% B: ACN with 0.004% of Phosphoric Acid, 20 µL/min; [2]D: Phosphoric Acid 0.004% B: ACN with 0.004% of Phosphoric Acid, 3.4 mL/min; **Theoretical Peak Capacity:** 3468 **Practical Peak Capacity:** 1850	[33]
White Wine and Orange juice	Low-molar-mass organic acids	SCX×RPLC	UV (210 nm)	**Mobile phase:** [1]D: Potassium Hydroxide 1-50 mM, 100 µL/min; [2]D: 20mM phosphate buffer with 10% of Methanol, 1.5 mL/min; **Correlation Coefficient of the two columns:** 0.0057	[67]

797 **Table 2.** *Cont.*

Sample	Analytes	Column Set	Detection	Observations	Reference
Mandarin essential oil	Carotenoids and Carotenoid Esters	NPLC×RPLC	DAD and MS	**Mobile phase:** ^{1}D: n-Hexane and Ethyl Alcohol (for Free Carotenoids) or n-Hexane/Butyl Acetate/Acetone (80:15:5) (for Carotenoid Esters), 10 µL/min; ^{2}D: 2-Propanol and Acetonitrile with 20% H_2O, 4.7 mL/min; **Theoretical Peak Capacity:** 986 and 651	[32]
Red Orange Essential Oil	Carotenoid	NPLC×RPLC	DAD and MS	**Mobile phase:** ^{1}D: n-Hexane/Butyl Acetate/Acetone (80:15:5) and n-Hexane, 10 µL/min; ^{2}D: 2-Propanol and Acetonitrile with 20% H_2O, 5 mL/min; **Theoretical Peak Capacity:** 551	[48]

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