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Comprehensive Two-Dimensional Liquid Chromatography Methods for Analysis of Polyphenols in Food Samples

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Abstract

Phenols and flavonoids presenting an enormous structural variability are receiving special interest because of their broad spectrum of pharmacological effects. The identification of these polyphenol derivatives in food samples is a difficult task due to the complexity of the structures and the limited standards commercially available. So far, one-dimensional chromatography was the most widely applied analytical approach for their analysis. However, when dealing with very complex media, a single separation system often does not provide sufficient resolving power for attaining rewarding results. Comprehensive two-dimensional Liquid Chromatography (LC×LC) is a technique of great analytical impact, since it offers much higher peak capacities than separations in a single dimension. The present review describes the applications carried out in the field of LC×LC for polyphenol separation in food and food-related products.

Keywords: Polyphenols; Food; Comprehensive liquid chromatography; Mass spectrometry

Abbreviations

LC: Liquid Chromatography; MS: Mass Spectrometry; LC×LC: Comprehensive Liquid Chromatography; ESI: Electro Spray Ionization; APCI: Atmospheric Pressure Chemical Ionization; GC: Gas Chromatography; CE: Capillary Electrophoresis

Introduction

Phenols and flavonoids presenting an enormous structural variability (5000 derivatives are known today), are receiving special interest because of their broad spectrum of pharmacological effects [1]. The identification of these polyphenol derivatives in food samples is a difficult task due to the complexity of the structures; from a chemical viewpoint, they can be classified in different groups, *i.e.* phenolic acids, flavan-3-ols, flavanones, flavones, flavonoids, lignans, and so on. To this regard, the most common separation techniques used to determine these kinds of bioactive compounds in such samples have been Capillary Electrophoresis (CE), Gas Chromatography (GC), and Liquid Chromatography (LC). CE provides high efficiencies in short migration times with small amounts of reagents and sample volumes needed [2], although its main disadvantage is the low concentration sensitivity. GC is the least used technique for this purpose, since a derivatization step is necessary. LC in combination with Mass Spectrometry (MS), using Atmospheric Pressure Chemical Ionization (APCI) and Electro Spray Ionization (ESI) as interfaces under positive and negative ionization modes, is usually adopted, for identification and structural characterization of these compounds. However, when the polyphenol content is very complex, one-dimensional LC is not sufficient for attaining rewarding results. As a consequence, to overcome such a limitation multidimensional Liquid Chromatography (2D-LC) techniques, where the two dimensions are based on different separation mechanisms, could be exploited [3-6]. Multidimensional LC (MD-LC) techniques can be classified into "heart-cutting" and "comprehensive LC", abbreviated as LC-LC and LC×LC respectively, according to the well-known established notations [7]. In both approaches, the columns are connected by means of a switching valve. The main difference between these two techniques is the amount of the primary column effluent that is transferred. In the former approach, only a few selected fractions of the effluent, containing the analytes of interest are directed from the primary to the secondary column (dimension) [8,9]; in the LC×LC, the entire sample is subjected to separation in both dimensions [10]. The first LC×LC application can be dated back to 1978 thanks to the pioneeristic work by Erni e and Frei [11]; since then, especially in the last decade, an ever continuous increase of papers have been published in this field to suit dedicated case studies and some of them were devoted to polyphenol analysis [12-42].

LC×LC: General aspects and method development

2D-LC methods can be operated either under "off-line" or "on-line" mode depending on the way to transfer the first dimension (^{1}D) effluent to the second column (^{2}D). In the former case, fractions of the ^{1}D effluent are collected manually or *via* a fraction collector, and afterwards re-injected on the ^{2}D column. "Off-line" approaches are time consuming, difficult to automate, non-reproducible and prone to sample loss and contamination and artefact formation. "On-line" approaches are faster and more reproducible, but more difficult to operate because of the use of special interfaces. Whatever "off-line" or on-line" approach employed, a 2D-LC separation is considered "orthogonal" if the two separation mechanisms provide complementary selectivity [43]. Orthogonal separations can be achieved when suitable mobile and stationary phases are selected,

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taking into account the physicochemical properties of the sample components including size and charge, hydrophobicity and polarity. In particular, LC techniques offer a wide variety of separation mechanisms, such as Normal Phase (NP), Reversed Phase (RP), Size Exclusion (SEC), Ion Exchange (IEX) or Affinity Chromatography (AC), characterized by different selectivities.

The development and optimization of an LC×LC method requires the adjustment of many parameters. An LC×LC system is composed of at least two pumps, two columns, an injector, an interface and a detector. The interface has the function to hyphen the two dimensions and in the most common set-up, small volume fractions of the effluent from the ^{1}D are transferred *via* a multi-port switching valve into the ^{2}D. Before coupling, the methods in both dimensions should be optimized, considering the sample characteristics and the parameters that affect the peak capacity. For the ^{2}D analyses, separation time should be fast enough to ensure both complete fraction elution before the subsequent transfer and adequate ^{1}D sampling [44]; this time is slightly enhanced if a regeneration step needs to be considered when running gradient programs. Analysis speed in the ^{2}D, can be increased in different ways. For polyphenol analysis, monolithic columns were the first to be used, due to their short regeneration characteristics and high permeability, thus allowing operation at high flow rates without loss in resolution [12,16-18]. Another way to speed up the ^{2}D analysis is to use conventional columns packed with stationary phases of reduced particle size such as partially or superficially porous stationary phases [19-25] or sub-2μm particle packed columns [33]; in the latter case, a more sophisticated hardware capable of withstanding such high pressures (P_{MAX}= 440 bar) *viz.* Ultra High Pressure Liquid Chromatography (UHPLC) is required [33]. To speed up the ^{2}D analysis, the use of elevated temperatures in the ^{2}D have been also exploited aiming to strongly increase the flow rate thanks to the reduced mobile phase viscosity, even though the stability of the stationary phase and analytes must be taken into account [14].

Both dimensions can be operated under either isocratic or gradient conditions. As an interface for the automatic transfer of the ^{1}D effluent to the ^{2}D, many configurations have been proposed. The most widely used interface involves the use of a 2-position/10-port

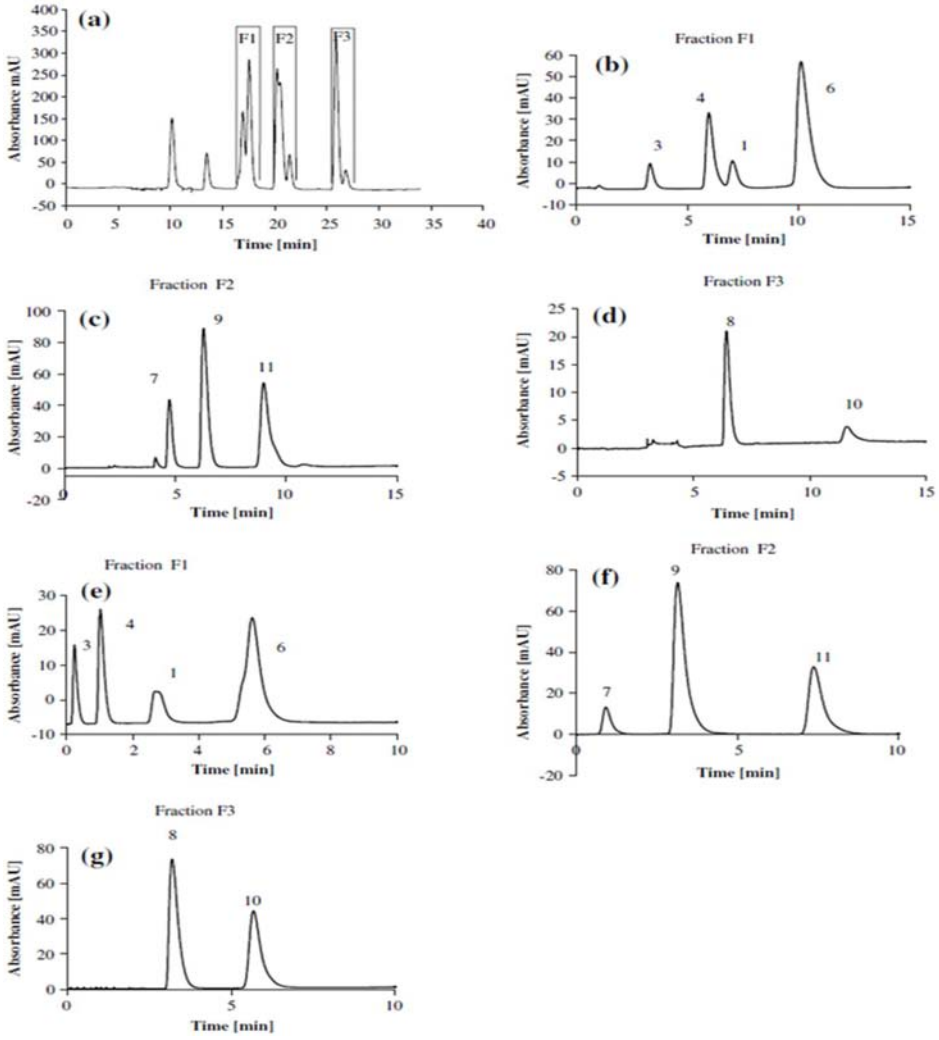

Figure 1: Heart-cutting of a mixture of phenolic antioxidants on a Purosphere Star RP-18e column (250x4.6 mm; 5 μm) in the first dimension and on a ZR-Carbon column (50x2.1 mm; 5 μm) in the second dimension. For details see ref [15].

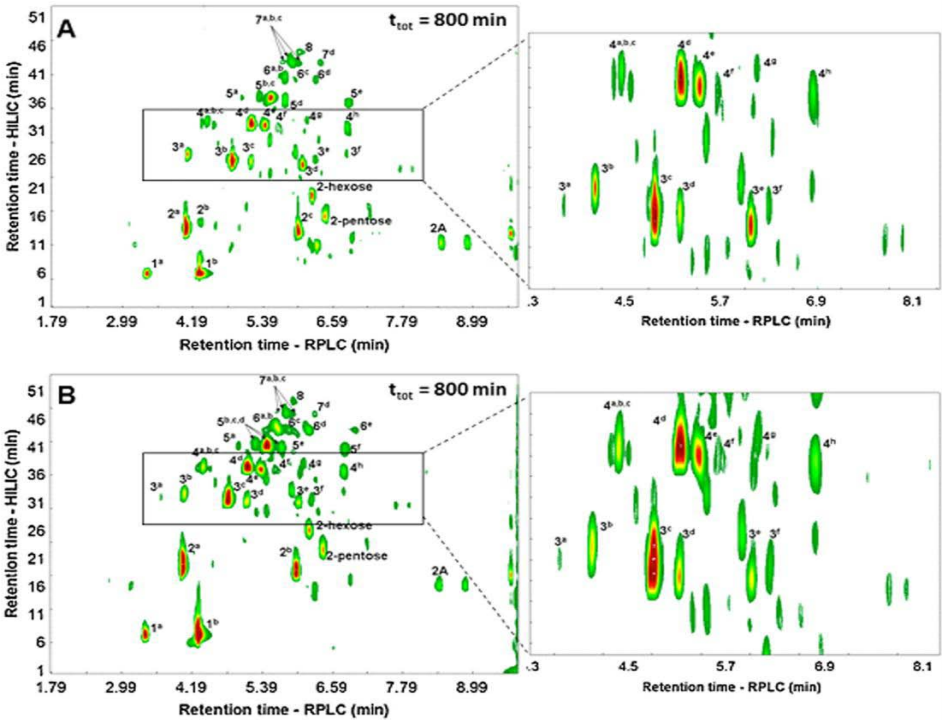

Figure 2: Fluorescence contour plots obtained for the HILIC × RP-LC analysis of a cocoa extract using off-line (A) and stop-flow (B) configurations. Peak numbers indicate the Degree of Polymerisation (DP) of PC isomers and letters differentiate isomers of the same DP. For details see ref [41].

switching valve [12-37], despite also two 2-position/6-port switching valves have been used as well [31,33,42]. The loops are alternately emptied and filled with the ^{1}D effluent prior to the ^{2}D analysis in a "continuous" way. The empty storage loops can be replaced by loops packed with stationary phase. Under these conditions, the ^{1}D mobile phase is preferably a weak solvent and the solutes are focused in the loop prior to be transferred to the ^{2}D; fast desorption by a strong solvent is afterwards performed by the ^{2}D mobile phase [12,16]. Also, two parallel ^{2}D columns have been employed in absence of storage loops using columns of the same batch and adapted tubing system [14,15,29]. As far as columns are concerned, the ^{1}D consists usually of micro (1.0 mm ID)- or narrow-bore (2.1 mm ID) columns providing flow rates compatible with conventional 4.6 mm i.d. columns in the ^{2}D. An issue of utmost importance is the compatibility of the mobile phases used in the two dimensions. The mobile phase eluting from the ^{1}D column should preferably consist of a weak solvent constituent of the ^{2}D mobile phase, in order to create a satisfactory peak compression thus achieving "peak focusing". Such a requirement is a must whatever combination of LC mobile phases is employed, especially if the solvents or solvent mixtures are not completely miscible. In the case of polyphenol analysis being most RP-LC×RP-LC separations, fully compatible 2D solvents are employed. A recent promising column combination in LC×LC employs the use of Hydrophilic Interaction Liquid Chromatography (HILIC) and RP conditions in the ^{1}D and ^{2}D, respectively. In the last two separation mode combination, the mobile phase used in the ^{1}D has a higher elution strength than the one used in the ^{2}D. For such a reason, the employment of micro-flow-rates in the ^{1}D is highly beneficial in order to minimize dilution and provide flow rates compatible with ^{2}D injection volumes [36-41].

LC×LC: Instrumentation and data handling

Commercial ready-to-use LC×LC systems are currently available from various manufactures, making the methodology much easier for practical uses, allowing matching specific 2D-LC requests. All conventional LC detectors, such as UV Photo-Diode-Array (PDA), Mass Spectrometric (MS) and Evaporative Light Scattering (ELS) detectors can be used in LC×LC. Usually, a single detector is installed after the ^{2}D column, although an additional detector can be used to collect the ^{1}D data, with the ^{1}D separation monitored only during the optimization step. The high speed of the ^{2}D analysis requires a very fast detector acquisition rate to ensure the adequate sampling which is critical for quantification purposes otherwise loss in resolution, due by a low number of data points, may occur. MS detection can be considered a third dimension to the LC×LC system, since the MS can be capable of identifying the co-eluting non-isobaric peaks when they are not resolved by chromatography. ESI and APCI MS interfaces receiving the ^{2}D effluent are preferably used for effective ionization of all polyphenolic compounds. For both visualization and quantification of 2D data, at least two commercially available software, specially designed are available on the market (http://www.chromaleont.it/chromsquare.html; http://www.gcimage.com/lcxlc/).

Comprehensive LC techniques for polyphenol separation in food analysis

On-line LC-LC have been successfully used for analysis of food polyphenols in only two works [15,45]. In the first case, natural polyphenolic antioxidants were separated using a C18 column in the ^{1}D and two parallel Zirconia Carbon columns working in alternating cycles in the ^{2}D. The combination of the two columns, run under temperature and solvent gradients, provided great differences in

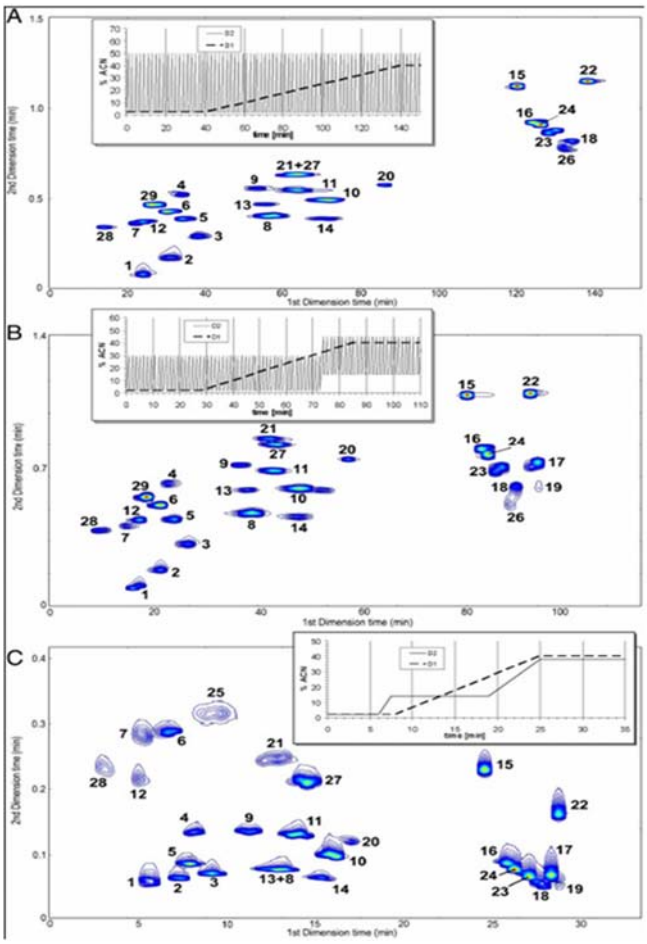

Figure 3: 2-D comprehensive LC×LC separation of phenolic acids and flavones on a PEG column in the first dimension and an Ascentis Express C18 column in the second dimension. (A) Parallel gradients with the FIF second-dimension gradient 1; (B) parallel gradients with the SIF second-dimension gradient 2; (C) parallel gradients with the CS second-dimension gradient 3 For details see ref [24].

separation selectivity in each dimension and an almost orthogonal 2D-LC system. Temperature gradients provided shorter separation times in comparison with solvent gradients (Figure 1). Such an approach was applied to the analysis of beer and wine samples. The more recent example was investigated for simultaneously determination of 12 major components, namely seven flavonoids, four phenylpropanoid glycosides, and N-trans-feruloyltyramine in tartary buckwheat.

As far as comprehensive LC approaches are concerned, both HILIC×RP-LC and RP-LC×RP-LC set-up by using different stationary phase chemistries have been employed for analysis of polyphenols in various food-related products [12-24,30,32,36-42].

HILIC×RP-LC combinations have been investigated for polyphenol analysis in beverages and plant extracts [30,32,36-41], allowing to separate compounds based on polarity and hydrophobicity, respectively. Such an approach have been used for elucidation of Phenolics and Procyanidins (PC) in apples and cocoa samples [30,40,41] and tea [32,36] extracts. The first application was reported for separation of phenolic compounds in apples and cocoa samples where an off-line LC×LC system was designed based on HILIC with a diol stationary phase and RP-LC conditions in the ^{1}D and ^{2}D, respectively. As detection, fluorescence, diode-array and negative ESI-MS systems, were employed for unravelling of the complex composition of low molecular weight phenolic compounds. The HILIC×RP-LC system was characterized by a very high practical peak capacity (over 2000) due to the low degree of correlation (R^2<0.2) between the selected separation mechanisms. The proposed methodology demonstrated its suitability for the analysis of various groups of phenolic compounds including proanthocyanidins, phenolic acids, flavonols and flavonol derivatives, all of which cannot be separated in a single analysis by conventional 1D-LC methods. More recently, three different LC×LC configurations namely on-line, off-line, or stop-flow were compared in terms of practical peak capacities, analysis times and peak production rates [40]. In a separate contribution, the experimental verification of the findings of this study was reported for analysis of cocoa procyanidins [41]. The results showed that while optimisation procedures based on theoretical considerations remain largely valid in practice, several important experimental considerations had to be taken into account to achieve maximum performance in all three modes of HILIC×RP-LC. On the one hand, the on-line analysis provided an effective tool for the screening of procyanidins content within reasonable times, whereas on the other, off-line- and stop-flow HILIC×RP-LC analyses were more suited for the detailed analysis of complex procyanidin

fractions (Figure 2). In particular, stop-flow operation had a negligible effect on the ^{1}D band broadening under the optimised experimental conditions used. Finally, off-line and stop-flow analyses provided much higher, and similar, resolving power at peak capacity production rates roughly half those obtained by the on-line system; however, the stop-flow system required the use of additional hardware and was experimentally more complicated but on the other hand it offered the advantages of complete automation and minor risk of sample alteration. Similar set-up for PC analysis was also reported for grape seeds extracts [38,39]. The investigated HILIC×RP-LC separation, followed by PDA and tandem MS detection, allowed the tentative identification of 43 flavan-3-ols, including monomers and procyanidins oligomers till a polymerization degree of 7 units with different galloylation degrees. The same set-up was also employed for the Phenolics profiling of different apple varieties in a frame time of less than 50 min, allowing the tentative identification of ca. 65 compounds on each studied sample, including flavan-3-ol oligomers up to a DP = 8, dihydrochalcones, flavonols and phenolic acids [39]. Such a study opened new possibilities for analysis of target and non-target metabolomics-related studies. As ^{1}D of a comprehensive HILIC×RP for separation of polyphenolic compounds, several polar stationary phases namely PEG, DIOL, Amide, Phenyl and sulfobetaine were compared with gradients of decreasing concentration of acetonitrile in buffered aqueous-organic mobile phases, and subsequently coupled on-line with short non-polar or weakly polar monolithic or porous shell columns in the ^{2}D, with fast RP-LC analyses (1-2 min) [37]. For optimum performance of HILIC×RP systems, micro-bore or capillary columns and low flow-rates were used in the ^{1}D, whereas short (3 or 5 cm) core-shell columns with larger diameters was tested at high flow-rates in the ^{2}D. 5-cm ^{2}D columns allowed the transfer of larger fraction volumes to the ^{2}D with respect to the 3-cm columns, without significant band broadening. For ^{1}D a new monolithic sulfobetaine polymethacrylate capillary column was tested under HILIC conditions, providing better orthogonality and good efficiency especially for low molecular compounds; moreover, the 0.53 mm I.D. allowed decreasing the fraction volumes close to the optimum, for separations within 1 or 2 min fast gradients in the ^{2}D.

On the other hand, RP-LC×RP-LC methods have been the most widely applied to the analysis of antioxidants and in particular polyphenols occurring in several types of food samples, mainly beverages and plant extracts [12-23,24,31,33,35,42]. However, in almost all RP-LC×RP-LC approaches investigated, peak capacity values were significantly lower with respect to the theoretical ones as a result of the coupling of partially correlated systems. A potential solution to overcome such problem was demonstrated by carefully choosing the LC gradient profile [24]. In particular various types of ^{2}D gradients in LC×LC were compared, namely "full in fraction", "segment in fraction" and "continuously shifting" gradients. Figure 3 shows the LC×LC separations of phenolic acids and flavones on a PEG column in the ^{1}D and two types of partially porous C18 columns in the ^{2}D (Ascentis Express and Kinetex) by using such gradients are illustrated. The effects of the gradient type on the bandwidths, theoretical peak capacity, and separation time and column pressure in the ^{2}D were investigated. A careful design of ^{2}D gradients could be a valuable tool when partially correlated systems are employed in LC×LC separations.

Conclusion

Comprehensive LC is a valuable tool for analysis of complex food samples. The popularity of such an approach increased with the availability of ready-to-use LC×LC systems equipped with dedicated software for both method development and data handling allowing qualitative and quantitative purposes.

For polyphenol analysis the coupling of hydrophilic interaction liquid chromatography and reversed phase conditions was the most efficient the very recent report describing the coupling of "partially correlated" RP×RP systems turned out to be a viable tool.

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