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Separation of Highly Complex Mixtures by Two-dimension Liquid Chromatography
(July 2007 to July 2013)

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The development of modern materials, increasing concerns for the safety of our environment and the healthy character of our foods, our improved understanding of the molecular interactions that take place in biological systems are posing new series of difficult analytical problems. Forty years ago, relevant analytical problems were solved by either separation or spectroscopic methods and the choice of the best method depended on the skills of the analyst and the tools available to him. Later, we learned to identify the most suitable method to solve given analytical problems. About twenty years ago, it became clear that new, difficult problems could be solved only by the coupling of a separation and a spectroscopic method. Successively, GC/MS, LC/MS, and, more recently, LC/NMR became widely used in application laboratories. It is clear now that hyphenated methods cannot solve many complex analytical problems. We need to develop more complex schemes than those permitted by the current implementations of classical methods. This explains why considerable attention is now devoted to the direct coupling of two separation methods and justifies the interest that multidimensional HPLC is attracting. The aim of our research was to study various possibilities available.

The goal of our research was a fundamental investigation of methods available for the coupling of two separate chromatographic separations that would considerably enhance the individual separation power of each of these two separations. This gain arises from the combination of two independent retention mechanisms, one of them separating the components that coelute on the other column, making possible the separation of many more compounds in a given time. The two separation mechanisms used must be very different. This is possible because many retention mechanisms are available, using different kinds of molecular interactions, hydrophobic or hydrophilic interactions, polar interactions, hydrogen bonding, complex formation, ionic interactions, steric exclusion. Two methods can be used, allowing separations to be performed in space (spreading the bands of sample components on a plate covered with stationary phase layer) or in time (eluting the sample components through a column and detecting the bands leaving the column). Both offer a wide variety of possible combinations and were studied.

1. Fundamentals of Multi-Dimensional Chromatography

The separation power of HPLC is ultimately limited by the difficulties encountered in preparing high efficiency columns and using them to separate complex mixtures [1-5]. Only multi-dimensional HPLC allow significant improvements of this situation.

1. Column Efficiency and Peak Capacity.

The separation power of HPLC columns is characterized by its efficiency or number of theoretical plates [1,6,7]. The important parameter in the study of complex separations,

however, is the actual number of peaks that the column can separate in a certain time or its peak capacity, the total number of peaks that can be lined up with a resolution unity, within a given time interval. This number is related to the column efficiency, to the retention window or distance between the first and last peak considered, and to the resolution required between the peaks [1,6,7]. Different equations apply in isocratic and gradient elution chromatography. In practice, under optimum conditions, the peak capacity of a column having an efficiency N is approximately $\sqrt{N}/2$ at a resolution unity in isocratic elution and slightly larger than 2 to 3 times $\sqrt{N}$ in gradient elution.

In principle, the peak capacity of a bi-dimensional separation scheme is equal to the product of the peak capacities in each direction [1]. In practice, it is not possible to couple two separations made in different dimensions without losing some separation power due to back-mixing during the transfer of the fractions from one dimension to the other and to a degree of correlation between the two retention mechanisms used.

2 - Statistics of Band Overlap.

The retention factors of the components of complex mixtures are randomly distributed, following approximately a Poisson distribution [8-10], result confirmed experimentally and extended to the distribution of the concentrations of the mixture components [11-13]. Consequently, it is difficult to separate a number of components comparable to the peak capacity. The statistics of band overlap [7-13] affords a simple equation that gives the probability P_m that an *m*-component mixture be separated into *m* bands with a resolution unity, on a column with a capacity n_c [13]:

$$P_m = e^{-\frac{m^2}{n_c}} \qquad (1)$$

This equation was extended to multi-dimensional systems. Felinger reviewed these issues [9].

The values of P_m given by Eq. 1 are surprisingly small. They decrease exponentially with increasing square of the number of components (m) and increase exponentially with increasing peak capacity (n_c). So, if the complexity of a mixture increases 10 times, the peak capacity of the column must increase 100 times to keep the resolution of the mixture constant. Analysts are resourceful and can do better than what predicts Eq. 1 and would be achieved with a random choice of phase systems. Yet, the difficulty of separating complex mixtures increases very fast with their increasing complexity.

2. Combinations of Two Uni-dimensional Separations.

All separations are due to selective transport that moves the molecules of the different components into different directions, to different places, or at different velocities along a given direction [10]. The scatter of the molecules of a component must be limited. Its degree is very important since it controls peak efficiency. The mass centers of the distributions of the molecules of the different sample components must be as remote as possible. The distribution patterns yielded by the separations in the two directions must

be correlated as little as possible.

The separations provided by the methods used in classical chromatography are made in either the time or the space dimension of the separation space. The separations made by gas chromatography (GC) or high performance liquid chromatography (HPLC) are time separations. Their theories are well known [1,5,10]. The separations carried out in the space dimension use a thin porous layer as the separation space and, in contrast with separations made in time, the sample components are moved to different locations of this bed. At the end of the separation, the bed is scanned to locate the bands.

There are several possible combinations for multi-dimensional separations. First, two successive separations may take place in two time dimensions. This scheme is known as 2D column chromatography or 2D-HPLC. This advanced method became popular in the last few years as comprehensive 2D-HPLC. Alternately, two separations may take place in the same physical space, in two perpendicular directions. This method is known for 65 years as 2D thin layer chromatography or 2D-TLC [14,15]. It was widely used in chemical and biochemical laboratories during the second half of the last century but is now unknown from most chromatographers.

1 - Combination of Two Time Separations.

This combination can be implemented in three different ways [1]. (1) In On-line 2D-LC, small fractions of eluent collected at the end of a first column are analyzed on-line on a second column while the next fraction is collected. (2) In stop-and-go 2D-LC, the stream flowing through the first-dimension column is stopped while the fraction just collected is analyzed on the second dimension column. This increases the analysis time and avoids the storage of collected fractions; and (3) In Off-line 2D-LC, the fractions eluted from the first column are stored and injected successively on the second column. It is important to keep in mind that time is the currency in which we pay for separation power. The total analysis time of the first method is practically the analysis time on the first dimension colimn; for the second method it is the elution time on the first column plus n times the difference between the duration of the second analysis and the elution time of each collected fraction. For the last method, it is the sum of the analysis time on the first column and n times the analysis time on the second column. The analysis time increases rapidly from approach 1 to approach 2 and 3. The peak capacity generated increases also rapidly.

The principle of real time comprehensive $LC^t \times LC^t$ demands that fractions eluted from the first column be analyzed on the second one during a time less than a quarter of their natural band width. The volume of each of these fractions analyzed on the second one must be equal to the standard deviation in volume unit of the first column [16].

$$\sigma_1 = t_2 \qquad (2)$$

This very short time places serious demands on the performance of the second column that must be very rapid and cannot deliver a high peak capacity. The same rule applies to other approaches of comprehensive LC but imposes less requirements since more time is available to perform the second analysis. The peak capacity of a column is

proportional to the square root of its plate number, with a proportionality coefficient function of the separation space available (i.e., of the range of values of k'). The peak capacity of 2D real time combinations is proportional to

$$\frac{t_1}{\sigma_1}\frac{t_2}{\sigma_2} = \frac{t_1}{\sigma_2} \qquad \textbf{(3)}$$

The proportionality coefficient depends on the sizes of the two separation spaces (and on whether elution is done under isocratic or gradient conditions. Equation 3 should be handled carefully because the column efficiency in gradient elution is not related to the ratio t_1/σ_1.

The first of the approaches listed above was selected by Schoenmakers et al. [17-19] to separate functional poly(methylmethacrylate) and other mixtures of copolymers. It combines an HPLC separation on silica and a SEC separation. The cycle time was 1 min. This approach was also used by Stoll and Carr et al. [4] for the separation of indole-3-acetic acid derivatives in maize seedlings, combining two successive separations on different packing materials, both performed in gradient elution. The cycle time in this case was 21 sec, a very short value made possible by running the second column at 110C. A total peak capacity of 900 was achieved in 25 min.

The second approach was selected by Bedani et al. [20] for the analysis of hydrolyzates of whey proteins, separated first with a 30 cm TSK GEL column eluted with water and then with a Chromolith monolithic silica column eluted with a gradient of acetonitrile. During the first elution, the eluent flows into the second column. Every 30 sec, the connection between the columns is closed, the flow though the first column stopped and gradient elution of the second column starts. The analysis time on the second column is 9 min, the total analysis time 500 min, for a peak capacity of 300.

The third approach, that of Yates [21-24], analyzed trypsin digests of protein mixtures and is known as MudPIT 2. It combined a series of 13 step gradients of eluent ionic strength on a strong cation-exchanger column (SCX). Each step releases a group of peptides separated by an acetonitrile gradient on an RPLC column [21-24] in a two hour analysis. The total analysis time exceeds one day, for a total peak capacity of about 6500, nearly independent of the efficiency of the SCX column.

Too few results are available in the literature to support general conclusions. That the peak capacity achieved by Bedani et al is lower than that reported by Carr is not a proof that Carr's approach is better. In fact, Carr's results illustrate as much the limitations of his approach as its advantages. A very high peak capacity was achieved but it required a considerable effort in the development of an exceptionally fast and efficient second column. This effort is difficult to replicate to solve any new problem. The optimization of the experimental conditions requires considerable attention and leads to markedly different approaches and solutions with the three options.

For example, Eq. 3 shows that the choice of the first option requires a first column that gives a broad overall resolution of the sample components (large retention time, t_1) and a second one that is highly efficient. The choice of alternate options leads to different

requirements. The need to store peaks before injecting then in a second column is not a serious drawback, particularly if the peaks are stored immobile in a column. Experience of peak parking shows that band broadening is small [25,26]. Axial diffusion is the only source of band broadening active and its contribution is modest.

2 - Separation of trypsin digests.

The third option was used to separate trypsin digests of cytochrom c. The first column was 110×4.6 mm and packed with Partisphere SCX, 5 µm (Whatman, Florham Park, NJ), a strong cation exchanger. It was eluted with a gradient of KCl in H_2O/acetonitrile. Fractions of the effluent were collected continuously, one 200 µL fraction every 20 sec, giving 140 fractions. These fractions were successively injected on a column packed of C18-bonded particles (Halo, AMT, Wilmington, DE), eluted with a gradient of acetonitrile in water, from 5 to 50% acetonitrile in 4 min, at a flow rate of 3 mL/min. Between two analysis, this column was regenerated for 4 min with an aqueous solution of acetonitrile. Figure 1 shows the separation as a plot of the signal intensity (in four color shades) versus the retention times on the two columns. The first separation has a peak capacity of ca. 50, the second a peak capacity of ca. 140, providing an overall peak capacity of 7000 [6]. The analysis time was 28 hours.

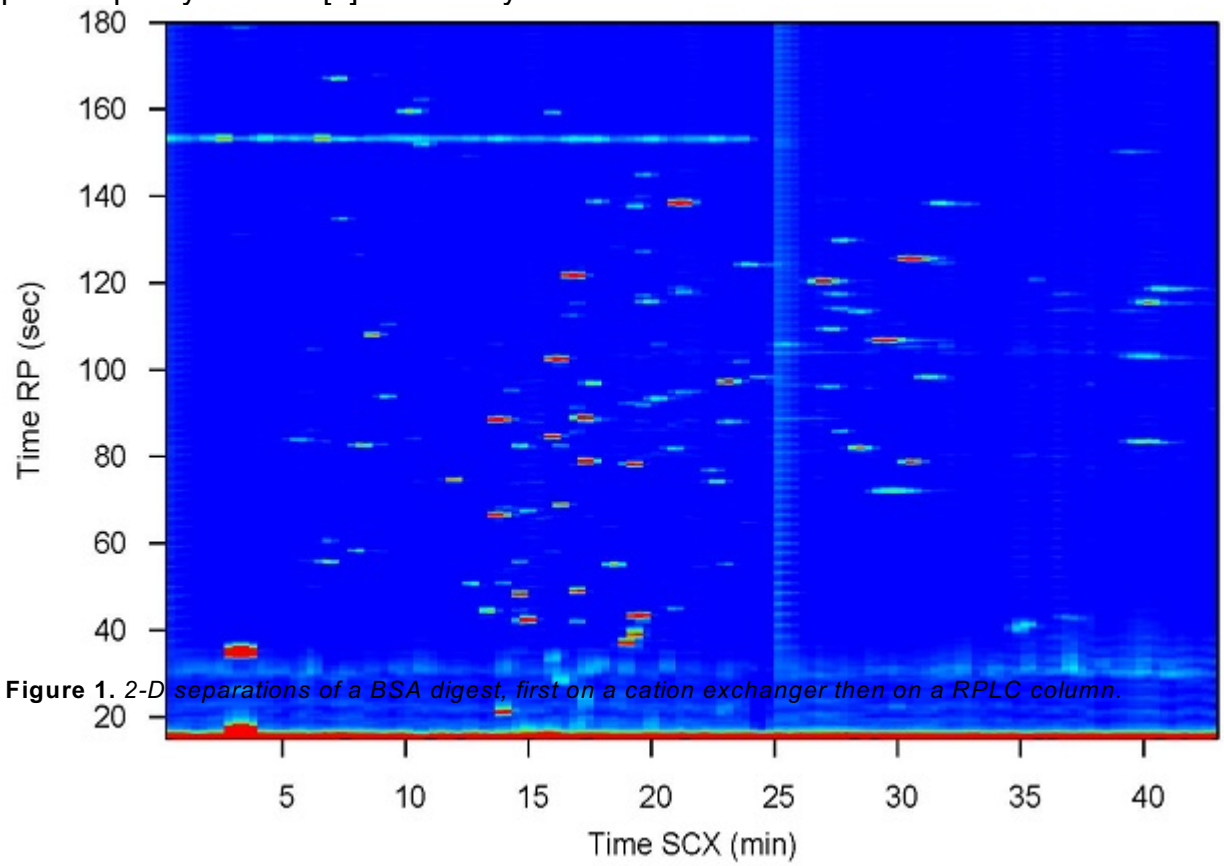


Figure 1. *2-D separations of a BSA digest, first on a cation exchanger then on a RPLC column.*

Due to the fundamental differences between the molecular interactions of the solutes and the two stationary phases, ion-exchange and reversed phase chromatography, the retention data of peptides on the two columns are poorly correlated, allowing full use of most of the separation space. Thus, the spots corresponding to the different peptides

separated are scattered over nearly the whole separation space in the Figure. The differences in the analysis times on the two columns and in their efficiencies explain the differences in the two sizes of each spot. The separation power achieved in Fig. 1 is an order of magnitude larger than that of most 2D analyses [19,20,27-29]. The resolution achieved is due to the selection of the very efficient Halo column for the second separation [30-32]. This increase in separation power comes at the cost of a large analysis time. However, a similar gain in separation power in 1D separations would require a nearly two orders of magnitude increase in the analysis time, making 2D-LC a most attractive solution. The only other method affording separation powers corresponding to peak capacities of a few thousands was Mudpit [33,34]. Our method is far from optimized yet. It provides a peak capacity approximately three times larger for nearly the same analysis time as Yates' method. Compared to Mudpit, the gain is due in part to the substitution of a continuous gradient to a step gradient program for the first separation, allowing the collection of many more fractions of the first column eluent. Although less powerful than the combination of two narrow bore columns described by Shen et al. [35] that was made possible by the use of a mass spectrometer as the detector, our method required also a shorter analysis time.

Figure 1 illustrates the separation power of off-line methods and the possibilities offered to analysts by the full palette of the implementations methods of multi-dimensional liquid chromatography that are now available [1]. The main value of our work is in showing how considerable improvements in peak capacity in 2D chromatography are possible at the cost of long analysis time. Even this approach, however, is insufficient to solve many complex analytical problems. A way to accelerate analyses would be to run simultaneously all second dimension separations. This requires the development of space-based not time-based separation methods [1].

3 - Optimization of Two-Dimensional Chromatographic Separations.

In all implementations of two-dimensional chromatography, the separation made on the second column must be repeated a large number of times. A detailed analysis of the different components of this cycle time was made for the three possible combinations of column chromatography, on-line, stop-and-go, and off-line. This allowed a comparison of their performance in terms of the peak capacity generated and the total analysis time. We showed that, for a given target peak capacity, there is an optimum capacity of the first dimension column and an optimum density of the fractions collected in the first dimension. In the case of the separation of protein digests, the best performance achieved were peak capacities of 266 in 20 minutes and of 4000 in 5 hours. The experimental results published confirmed these calculated performance [36,37].

4- Data acquisition and handling

Some of the most serious problems encountered in two-dimensional chromatography are related to the acquisition and handling of the detector signals. Chromatography separates complex mixtures but dilutes the sample components in the process [36]. Their dilution, which increases with increasing retention, is ca. two orders of magnitude in isocratic elution, somewhat lower in gradient elution [37]. So, it is critical to develop methods allowing detection of relatively weak signals and their accurate integration [36].

Due to the nature of comprehensive multi-dimensional HPLC, adjacent cut fractions of the second dimension chromatograms may contain compounds common to more than one cut fraction. We developed an algorithm to automatically recognize the different peaks eluted in the successive chromatograms recorded for the second dimension of a multidimensional separation that correspond to the same compound and to calculate their cumulative peak area [38]. The algorithm determines the components common in adjacent cuts and subsequently calculates the area of a two-dimensional peak profile by interpolating the surface of the 2D peaks between related peaks in adjacent fractions. This cumulative peak area provides an estimate of the volume of this peak in the 2D chromatogram, hence of the amount of the corresponding compound. To determine the retention times of the peaks eluted in the first and second dimension chromatograms, we used a Savitzky-Golay filter to smooth the first, second, and third derivatives of the experimental profiles. A close examination of the shapes of these curves informs on the number of peaks and provides initial values to fit theoretical profiles. This algorithm was tested by calculating the cumulative peak area of a series of 2D-HPLC separations of alkylbenzenes with varied concentrations. The relationship obtained between these concentrations and the cumulative peak area was good [38].

The areas, the first and the second moments of the peaks recorded in chromatography need to be calculated to determine the amounts of the corresponding compound in the sample analyzed (areas), their retention times (first moments), and the corresponding column efficiencies (second moments), These integrations are difficult because base lines are always noisy and the integration of the peaks should not begin too early nor end too late, otherwise the integral underestimates or overestimates the calculated moment. The determination of the integral boundaries are generally done by the data stations associated to modern chromatographs. These results are too important to be accepted on blind faith and it is necessary to check their validity. This is often done by visual examination of the chromatograms, which is time consuming but only moderately accurate. Several simple techniques have been elaborated for the identification of these boundaries. These methods provide significant reduction in the time needed to perform rapid automatic calculation of peak moments and to evaluate the quality of a separation while improving the accuracy of the estimated values of the column efficiencies. The identification of the peak boundaries as functions of the peak widths and examination of the slope of the plots of the signal to noise versus the time are viable alternatives to a manual determination [39]. To check the validity of our methods, we calculated the profiles of simulated bands based on the Gaussian and the EMG models and derived an automatic procedure for the calculation of their moments. This method was used to find relationships between the peak characteristics (notably the peak width and its tailing) and the parameters required for the automatic calculation of accurate statistical peak moments. Computers can be programmed to extract the key peak parameters from chromatograms and to select acceptable integration boundaries. This approach automatize this form of data analysis. We used real chromatographic peaks to test this procedure and reported the appropriate band boundaries [40].

3- New combinations of two different separations

During the last two years of the grant, we investigated the results obtained with different combinations of retention mechanisms and determined to which degree diverge the retention patterns that they provide for different chemical families. Classically, 2D-LC separations combine conventional reversed phase, normal phase, ion-exchange, and/or size-exclusion chromatography.

Mixed mode stationary phases can provide a secondary retention mechanism that adds a dimensionality to the surface of conventional high performance liquid chromatography adsorbents. Applying these interactions, we performed (2D)-HPLC separations of a β-lactoglobulin tryptic digest with a Scherzo SM-C_{18} column using the same mobile phase at pH=7 in the first and pH=2 in the second dimension [41]. The mechanism divergence was estimated by the peak capacity of the separation achieved and a geometric approach to factor analysis (GAPA [42]). The results obtained were better than those provided by combining a Scherzo SM-C_{18} and a classical C_{18} column.

Last year, we developed a novel separation method of comprehensive two-dimensional chromatographic by coupling liquid chromatography (HPLC) and supercritical fluid chromatography (SFC) [43]. This method consists in eluting the second column with a mobile phase rich in carbon dioxide. It differs markedly from HPLC due to the unusual properties of supercritical fluids. Their viscosity is five to ten times less than that of the solvents used in HPLC. Hence the diffusion coefficients of solutes are much higher and so are the optimum flow rates, making the separations very fast without causing any loss in the resolution achieved since the maximum column efficiency is nearly the same in HPLC and in SFC. The high compressibility of supercritical fluids means that the mobile phase density and the local pressure decrease rapidly along the column. Since the equilibrium constants of analytes between the stationary and the mobile phase, hence their retention factors depend on the local density, they also depend on the flow rate since pressure profiles along a column depend on the average flow rate This property renders the method difficult to understand and to control. We are studying these issues.

1- Separation of the components of a natural oil.

Our first application of SFC was the separation of blackberry sage fragrant oil, with HPLC in the first dimension and SFC in the second [43]. A RPLC column eluted with an ACN gradient was used in the HPLC dimension and an amino-bonded silica column eluted with ACN as the modifier in the SFC dimension. This separation was made in the off-line mode. Fractions eluted from the HPLC column were collected and successively injected in the SFC column. The correlation coefficient between the retention factors of analytes was -0.757, showing that the two modes of separation are very different. The peaks eluted first in HPLC are the last ones eluted in SFC and vice versa, hence the negative value of the correlation coefficient, a most unusual situation. The method provides a practical peak capacity of 2400 in 280 minutes, which is a very impressive value. The results demonstrated that coupling an HPLC and an SFC separation has great potential for two-dimensional chromatographic separations. This explains why we

persisted in using this combination, RPLCxSFC.

2- Separation of terpenic hydrocarbons.

We measured the retention patterns of terpene hydrocarbons on a pair of columns, using graphitized carbon black and a C_{18}-bonded stationary phase [43]. The patterns of retention factors are significantly different. The PGC surface provides an important selectivity toward the shape, polarity, and structure of the terpene hydrocarbons in the sample that is not afforded by the C_{18} surface. Plots of the retention factors similar to those typically used to represent 2D-HPLC separations confirm this observation. A geometrical method of factor analysis was used to measure the separation divergence, together with the selectivity and the product selectivity factors of closely related species (GAPA, ref. [42]). When methanol was used as the mobile phase for the PGC surface, a large fraction of the separation space could be utilized. That is further reflected by a spreading angle of 80.3°. The PGC material allowed resolving structural isomers while the C_{18} phase could not. It was also found that the choice of the mobile phase is important when using this material. A much larger degree of utilization of the separation space was observed with methanol than with acetonitrile that displayed a spreading angle of only 40.8°, barely twice smaller than for methanol.

3- Separation of switch grass extracts.

Switch-grass (*Panicum virgatum L.*) is a warm-season perennial grass native to North America, valued as an energy crop for biomass and biofuel production [44]. Dry grass samples were extracted and analyzed by off-line two-dimensional chromatography. The method combined a first dimension HPLC separation and a second dimension SFC separation. The first separation uses a 150×4.6 mm Ascentis Express RP-Amide column packed with 2.7 µm core-shell particles, eluted at 24°C and 1 mL/min with a 15 minutes, 5 to 100% gradient of ACN in water followed by a 5 minutes concentration hold in pure ACN. The SFC separation uses a 250×4.6 mm PrincetonSFC 2-Ethylpyridine column packed with 5 µm particles (100 Å pore size), eluted at 27°C, 180 bar back pressure and 4 mL/min, with a 5 min. 95 to 60% gradient of MeOH in carbon dioxide followed with a 4 minutes concentration hold at 60% MeOH and a 3 minutes regeneration. This combination affords a peak capacity in excess of 12,500 in 16 hours for the analysis of the switch-grass extracts. These separations are nearly perpendicular (spreading angle 87.7°), so 97% of the available separation space could be used, which provided a resolution speed of 13.0 peaks per minute.

4- Advantages of using an SFC separation in the second dimension

These last results confirm the great advantages that SFC provides when used for the second dimension in a two-dimensional separation method. Due to the high diffusion coefficients of analytes in supercritical fluids and to their low viscosity, SFC separations can be carried out with a mobile phase flow velocity that is several times faster than the velocity used in HPLC separations and yet it requires lower inlet pressures. It also provides retention patterns that are markedly different from those achieved in HPLC. This combination permits using a very large fraction of the separation space of a 2D chromatographic separation. Using much faster separations in the second dimension makes possible to use long columns, providing high efficiencies.

4. Applications of Two-Dimensional Chromatography to Separate Metabolites

Two-dimensional liquid chromatography has become widely used in the analysis of complex mixtures [19,20,28-30]. Its most important field of application is becoming in the analysis of complex mixtures of biochemical origin, particularly proteins and metabolites of various origins [22-25]. The objective and aims of our work were to apply the experience previously acquired in two-dimensional chromatography to separate the components of the lipids and lipid metabolome of strains of microalgae. The primary aim was to separate the various classes of lipids produced and to identify and analyze the most important components of these classes. The eventual goal was to investigate how the experimental conditions under which these algae are raised affect the algae dry weight, the composition and production rates of these lipids in order to provide tools allowing the assessment of the performance of strains of microalgae to produce suitable bio-fuels.

1- Strains of algae studied.

We made cultures of *Botryococcus Braunii* showa in a modified Bold Basal Medium (25g $NaNO_3$; 2.5 g $CaCl_2.2H_2O$; $MgSO_4.7H_2O$; 7.5 g; K_2HPO_4 7.5 g; KH_2PO_4 17.5 g; NaCl 2.5 g; EDTA 50 g; KOH 31.5 g; $FeSO_4.7H_2O$ 4.98 g; H_2SO_4 1.0 mL; H_3BO_3 11.4 g.; ZnSO4.7H2O 8.8 g; $MnCl_2.4H_2O$; 1.44 g; MoO_3 0.71 g; $CuSO_4.5H_2O$ 1.57 g; $Co(NO_3)_2$, $6H_2O$ 0.49 g), using $NaNO_3$ as the nitrogen source in the media. Two conditions were tested, nitrogen rich and deprived (No NaNO3), to affect the output of the strains. Trace elements were added to some cultures to test for their effects on oil production.

Botryococcus braunii is a unicellular green colonial micro-alga, which is widely distributed around the world in fresh and brackish water [45]. It shows significant promise as a bio-fuel candidate. Geochemical analysis of petroleum showed that ancestral strains of *Botryococcus Braunii we*re the source of up to 85% of the world's present petroleum reserves [46]. These cells accumulate three major types of hydrocarbons [47]. It exists as three main races. The A race produces fatty acid-derived alkadienes and alkatrienes; the B race produces triterpenes and some heavier branched alkenes known as the botryococcenes; the L race produces mostly the tetraterpene lycopadiene. The topic of this study was the B race.

The most common botryoccocene is a triterpene with a 22 carbon atom linear chain having 6 methyl groups and one unsaturated ethyl one. It is similar to that of squalene, which has a 24 carbon atom chain with six methyl groups. There is a large number of isomers and analogs. Small droplets of hydrocarbons that the alga cells synthesize are attached to the cells while most hydrocarbon compounds secreted impregnate them. The reason why these algae excrete the hydrocarbons is unknown but suspected to ensure the flotation of the colony on the surface of the ponds where it grows and, possibly, to protect it from competitors or scavengers.

2- Extraction of the lipid and lipid metabolite fractions.

At the end of the culture cycle, the algae were recovered by filtration, the sample

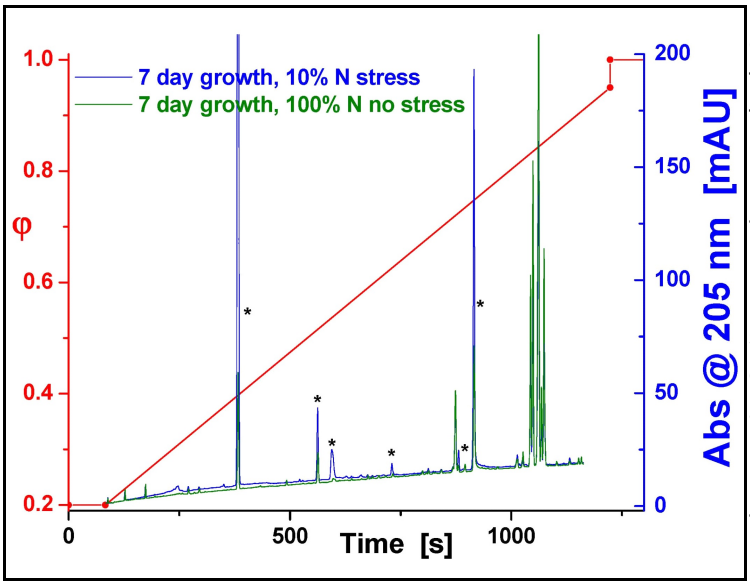
weighed and the dry weights recorded. The metabolites were extracted by centrifuging the cells and treating them with an aqueous solution of ACN and MeOH to remove cell debris by centrifugation and extracting the hydrocarbons [48]. The fractions recovered were analyzed by two-dimension liquid chromatography with UV spectrophotometry detection. During these analyses, we realized that the samples contained hydrocarbons which, although having weak UV chromophores, were detected as small peaks, still easily observed due to the high concentration of these hydrocarbons.

3- Analysis of algae metabolites.

Off-line two-dimensional liquid chromatography with tandem mass spectrometry detection (2D-LC/MS-MS) was used to analyze the metabolomic species produced in a series of cell cultures. This method was developed earlier [49-51] and validated [49]. It combines (1) a 2D-LC separation, using two different columns and mobile phases selected for providing different elution patterns for the sample components; and (2) MS-MS analysis as the detection mode. For the second HPLC separation, a single set of conditions using successively the positive and the negative ionization modes in mass spectrometry allowed the detection of a total of 141 extracted metabolite species, with an overall peak capacity of ca. 2500 [49]. We selected a cation exchange and a hydrophilic interaction systems for our two-dimensional separation method. As planned, we pursued investigations on two-dimensional liquid chromatography during the first year of the project. We selected retention mechanisms appropriate for these separations, designed, implemented, optimized, and tested the association of several pairs of retention mechanisms, and investigated problems related to the quantitation of the compounds identified.

Although growth appeared to progress at a satisfactory rate, giving green solutions and a significant concentration of chlorophyl measured by fluorescence, the amount of dry weight extracted from our cultures was surprisingly low, due to a combination of too small volumes and low concentrations. The low amount of available extracted material limits the analytical work that could be done. From the solutions, we extracted lipids and lipid metabolites and found out significant amounts of hydrocarbons, methyl esters and other derivatives of fatty acids present in the algae, so decided on a second extraction step to analyze for these compounds, which is best done by GC/MS and by SFC.

The fraction of hydrocarbons produced by the algae was analyzed by HPLC using a 70 to 97% gradient of acetonitrile in water [52]. The gradient program was set so that the retention factor of the last eluted hydrocarbon was around 15 at the beginning of the gradient run and around 1.5 when it exited the column. This ensures that the elution of the botryococcenes takes place soon after the hold-up time, in contrast to what is usually observed in gas chromatography [52]. To spread the elution times over the whole gradient window, the elution time of the most retained hydrocarbons, which seems to be the most abundant, was adjusted at be one hold-up time shorter than the gradient time. Then, the mobile phase composition is abruptly increased to 100% acetonitrile and the column washed with four column volumes. This method provides a wide retention window, a large gain in the analysis time and low solvent consumption. This also maximizes the peak heights, hence minimizes the detection limits.



The concentrations of six of the compounds (see asterisks) in the figure increase by more than 100% for algae stressed by depletion by 90% of the nitrogen content in their nutrient. Other analyses showed that squalene is eluted after the last eluted analytes, therefore, none of the components present in significant concentration in the extract solution is squalene nor its analogs. Most likely, the molecular masses of the hydrocarbons present in the extract are smaller than that of squalene (410 g/mol), probably around 300 for the most retained ones. The retention times of these hydrocarbons was comparable to that of the polycyclic aromatic hydrocarbon naphtho[2,3-a]pyrene, which has a molecular weight of 302 g/mol. Therefore, one can expect that the hydrocarbons detected contain about 20-25 carbon atoms.

We were able to identify several branched alkanes or alkenes, possibly botryococcene (but this needs confirmation), which had already been identified in the cultures of other related algae strains and several fatty acid methyl esters in the C16 to C18 group. The systematic identification of these components would require their MS analysis. The addition of trace elements enhances the production of algae; the lack of nitrogen inhibits it markedly as does the use of acidic growth medium, but to a moderate degree.

4- Production of algae metabolites.

The production of algae was estimated by measuring daily the fluorescence light collected at 685 nm upon irradiation at 450 nm of the cultures, using a fluorometer. After 10 days (in a few cases, after a month), the cultures were filtered and the weight of the dry mass collected was measured. The results in Table 3 are the averages of four measurements.

5- Investigation of reference compounds by HPLC/MS.

Authentic lipid standards were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Avanti Polar Lipids (Alabaster, AL, USA).

Chromatographic analyses were made with a 3000 Ultimate LC system, using a Kinetex (Phenomenex, Torrance, CA, USA) HILIC column at 30°C, in gradient elution. The gradient used (A) a 10 mM aqueous solution of ammonium formate at pH=3; and (B) a 10 mM solution of ammonium formate in H_2O/acetonitrile. This analysis provides a separation of the different lipid classes. Samples of the eluate were injected into the source of the Exactive Plus Orbitrap mass spectrometer using an electrospray ionization source (Thermo Fisher Scientific, San Jose, CA, USA).

The head groups in phospholipids and choline-related compounds are polar. Hydrophilic interaction chromatography (HILIC) is a convenient separation mode and acetonitrile the most appropriate solvent. Volatile ammonium formate or acetate are used as ionic additives to the mobile phases in LC/MS. When an electrospray ionization (ESI) source is used, additive concentrations above 10 mM should be avoided, due to detrimental effects on the detection sensitivity.

In our analyses, a 10 mM ammonium formate solution adjusted to pH=3 was used as the mobile phase additive. The gradient was optimized based on the retention data of reference compounds eluted under isocratic conditions, with a 10 mM solution of acetonitrile and ammonium formate. Buffering ACN improved the peak shapes of compounds with low retention factors. HILIC retention mechanisms are based on the adsorption of solutes on the stationary phase and/or on the partitioning of these solutes between the water layer formed on the adsorbent surface and the mobile phase. These interactions are due to electrostatic forces.

Unfortunately, the new mass spectrometer acquired by the of organic chemistry group was delivered, installed, and became operational only in late Spring 2013. We were unable to synchronize the operations of this instrument and of our SFC chromatograph within the limited time available before the end of the grant. So no LC/MS analysis could be done.

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ANNEX

LIST of 2009-2013 PUBLICATIONS SUPPORTED by GRANT DE-SC0001014

(1) Approaches to Comprehensive Two-Dimensional Liquid Chromatography Systems. J. N. Fairchild, K. Horváth and G. Guiochon.*J. Chromatogr. A,* **2009**, *1216,* 1363–1371.

(2) Optimization of on-line two-dimensional chromatography. K. Horváth, J. Fairchild and G. Guiochon. *Anal. Chem.*, **2009**, *81*, 3879–3888.

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