Focusing and non-focusing modulation strategies for the improvement of on-line two-dimensional hydrophilic interaction chromatography × reversed phase profiling of complex food samples.

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ABSTRACT.

Comprehensive two-dimensional liquid chromatography (LC × LC) is ever gaining interest in food analysis, as often, food-related samples are too complex to be analyzed through one-dimensional approaches. The use of hydrophilic interaction chromatography (HILIC) combined with reversed phase (RP) separations has already been demonstrated as a very orthogonal combination, which allows attaining increased resolving power. However, this coupling encompasses different analytical challenges, mainly related to the important solvent strength mismatch between the two dimensions, besides those common to every LC × LC method. In the present contribution, different strategies are proposed and compared to further increase HILIC × RP method performance for the analysis of complex food samples, using licorice as a model sample. The influence of different parameters in non-focusing modulation methods based on sampling loops, as well as under focusing modulation, through the use of trapping columns in the interface and through active modulation procedures are studied in order to produce resolving power and sensitivity gains. Although the use of a dilution strategy using sampling loops as well as the highest possible first dimension sampling rate allowed significant improvements on resolution, focusing modulation produced significant gains also in peak capacity and sensitivity. Overall, the obtained results demonstrate the great applicability and potential that active modulation may have for the analysis of complex food samples, such as licorice, by HILIC × RP.

Keywords: Metabolite profiling, two-dimensional LC, licorice, active modulation, trapping columns, resolution.

1. INTRODUCTION

The use of multidimensional liquid chromatography (MDLC) within the food analysis field is gaining interest, as foods and food-related products are normally considered as very complex matrices [1]. Indeed, it is frequent to find food samples that are simply too complex to be analyzed by conventional one-dimensional chromatography. In other cases, food-related samples may not be so complex in terms of number of compounds present, but these could be composed by mixtures of closely related components that are also difficult to be resolved. Although there are several approaches to MDLC of food, the use of comprehensive two-dimensional liquid chromatography (LC × LC) coupled on-line, presents different advantages over off-line modes as well as over other couplings, such as heart-cutting two-dimensional LC. Most-notably, faster separations may be obtained with high resolving power in a fully-automated way, thus, increasing robustness and reproducibility [2,3]. However, the optimization of a LC × LC method is far from being easy, as there are different inter-related parameters which modification may directly influence others [4]. These optimization challenges are even more pronounced when orthogonal separation mechanisms are coupled, which in practice, is the most-interesting approach. By selecting two independent non-correlated separation modes in both dimensions, significant gains on resolving power and peak capacity are potentially attainable. However, using two very different separation mechanisms in both dimensions means that important solvent incompatibility and/or immiscibility problems may be found. The combination between hydrophilic interaction chromatography (HILIC) in the first dimension (^{1}D) and reversed phase (RP) in the second dimension (^{2}D) has been shown to be characterized by a high degree of orthogonality for the analysis of complex food samples [5], providing with complementary retention. Although in these two separation modes the same types of mobile phases are employed, their coupling can be

termed as fairly incompatible, considering that the relative solvent strength is the opposite in each mode, thus, producing serious solvent mismatch.

In a LC × LC system, both dimensions are physically connected through the modulator. The most-widely employed modulator so far is based on the use of one or more switching valves equipped with two identical volume sampling loops [6]. This configuration allows the effective collection and injection of discrete ^{1}D effluent fractions into the ^{2}D continuously, by alternating the position and function of the two sampling loops. To translate this into practice, different analytical conditions should be established, mainly: i) a ^{1}D slow separation based on the use of very low flow rates, in order to minimize, as much as possible, the effluent fraction volume collected, and; ii) a fast ^{2}D using very high flow rates, in order to achieve fast separations in the shortest possible analysis time to allow a high ^{1}D sampling rate. As a consequence, set-ups involving the use of microbore columns in the ^{1}D combined with short wider columns (e.g., 4.6 mm i.d.) in the ^{2}D have provided good results [5]. This type of coupling implies the additional advantage of injecting relatively small volumes of ^{1}D effluent on the ^{2}D, thus, reducing possible band broadening. However, the main limitation directly related to the application of this approach is the characteristic low sensitivity obtained in LC × LC compared to regular one-dimensional methods, although potential deleterious issues due to solvent mismatch are significantly reduced [7].

To partially alleviate these problems, different modulators have been designed; among them, thermal modulators are included. Within this group, several improvements have been presented, such as a vacuum-assisted evaporation interface aimed to remove the solvent from the ^{1}D effluent prior transfer to the ^{2}D [8], or the development of an on-column thermal modulation device [9]. This latter device was shown to be able to apply heating and cooling cycles to capture and elute analytes to the ^{2}D producing narrower

95 bands. However, due to their sophisticated and complicated design, these thermal
96 modulators have not been to date extended to other applications. In parallel, new
97 approaches have been explored taking advantage of the higher robustness and simplicity
98 of valve-based modulation, such as the use of two parallel ^{2}D columns [ref 10]. Another
99 interesting possibility to enhance the performance is to substitute the regular sampling
100 loops by trapping columns [10-12]. By using this approach, analytes are adsorbed by the
101 stationary phase of the trap, typically with similar selectivity to that found in the ^{2}D,
102 during the collection position, and are then eluted by the ^{2}D mobile phase in the injection
103 position. Although, theoretically, the injection in ^{2}D mobile phase could also help to
104 produce narrow bands and even focusing at the top of the ^{2}D column, there still may exist
105 solvent incompatibility issues that may imply that not all the analytes contained in the ^{1}D
106 effluent are efficiently retained in the trap. To overcome this issue, a modulation
107 procedure termed "active modulation" has recently been reported [13]; this approach is
108 based on the introduction of a make-up flow of a weaker solvent after ^{1}D separation and
109 before entrance to the trapping column. This way, a reduction in the solvent strength is
110 fostered, increasing the retention of the trap towards the compounds separated in the ^{1}D.
111 Subsequently, when the valve is actuated, those retained analytes can be eluted from the
112 trap in narrow bands thanks to the ^{2}D mobile phase. From this basic procedure, other
113 modifications can be performed in order not only to improve the transfer of ^{1}D effluent
114 to the ^{2}D, but also to increase sensitivity and decrease analysis time. Although this active
115 modulation approach retains a high potential for the analysis of complex samples, its
116 applicability to food samples is still not demonstrated.
117 For this reason, the goal of the present work is to explore new possibilities to improve the
118 separation of complex food samples, looking for quantitative improvements on resolving
119 power, avoiding ^{2}D band broadening, as well as on sensitivity, using licorice as model

120 matrix. To this aim, different modifications at the modulator level are tested and
121 compared, studying their applicability on a HILIC × RP coupling. The influence of the
122 separation and modulation parameters applied on the separation and detection of the
123 secondary metabolite profile of licorice, previously developed in our lab [14], including
124 glycosylated flavanones and chalcones and other polyphenols as well as triterpene
125 saponins, is evaluated.
126
127 **2. MATERIALS AND METHODS.**
128 **2.1. Samples and chemicals.**
129 Licorice samples (*Glycyrrhiza glabra*) from the region of Calabria, Italy, were collected
130 in July 2015 and supplied from a local producer. For the extraction of secondary
131 metabolites from this sample, a simple procedure based on solid-liquid extraction assisted
132 by ultrasounds extraction was followed, as described before [15]. The extraction solvent
133 was a binary mixture ethanol/water (1:1, v/v) using a sample-to-solvent ratio 1:5 (w/v)
134 during 60 min. The resulting extract was filtered and evaporated to dryness. Prior
135 injection, the extract was redissolved in water/acetonitrile (3:7, v/v).
136 HPLC grade ethanol and acetonitrile were purchased from VWR Chemicals (Barcelona,
137 Spain) whereas ultrapure water was produced from a Milli-Q instrument (Millipore,
138 Billerica, MA). Acetic and formic acids were supplied from Sigma-Aldrich (Madrid,
139 Spain), while ammonium acetate was from Panreac (Barcelona, Spain).
140
141 **2.2. Instrumentation.**
142 The LC × LC-DAD instrumentation consisted on a first dimension (^{1}D) composed by an
143 Agilent 1200 series liquid chromatograph (Agilent Technologies, Santa Clara, CA)
144 equipped with an autosampler. A Protecol flow-splitter (SGE Analytical Science, Milton

145 Keynes, UK) was installed between the ^{1}D pumps and the autosampler in order to
146 minimize the gradient delay volume of the pump and to obtain more reproducible low
147 flow rates. The second dimension (^{2}D) was composed by an additional LC pump (Agilent
148 1290 Infinity). Both dimensions were connected by an electronically-controlled two-
149 position ten-port switching valve (Rheodyne, Rohnert Park, CA, USA) acting as
150 modulator equipped with two identical sampling loops or trap columns, as indicated. A
151 diode array detector was coupled after the second dimension in order to register every ^{2}D
152 analysis. The system was simultaneously controlled by two different PC running
153 appropriate ChemStation software; one controlled the ^{1}D, the autosampler and DAD,
154 whereas the other controlled the ^{2}D and actuated the switching valve. For the separations
155 involving the use of a make-up flow, a third LC pump (Agilent 1200 Series) was
156 connected through a t-piece between the outlet of ^{1}D and the switching valve. The used
157 additional make-up flow was delivered at five-, seven- and nine-times the ^{1}D flow rate,
158 as indicated.
159 The LC linear chromatograms were elaborated and visualized as 2D- and 3D-plots using
160 LC Image software (version 1.0, Zoex Corp., Houston, TX).
161

162 **2.3. HILIC × RP separation conditions.**

163 Different conditions and column combinations were employed during this research, as
164 described in Section 3. The common analytical conditions for each column used in the ^{1}D
165 were the following:
166 i) SeQuant ZIC-HILIC (150 × 1 mm, 3.5 µm, Merck, Darmstadt, Germany) column,
167 eluted using (A) acetonitrile and (B) 10 mM ammonium acetate at pH 5.0 as mobile
168 phases, according to the following gradient: 0 min, 3% B; 5 min, 3% B; 10 min, 5% B;
169 15 min, 10% B; 30 min, 20% B; 40 min, 20% B; 50 min, 30% B; 60 min, 30% B; 65 min,

40% B; 80 min, 40% B. The injection volume was 5 µL and the flow rate was set at 15 µL min^{-1}.

ii) ZIC-HILIC (250 × 2.1 mm, 3.5 µm, Merck, Darmstadt, Germany) column, eluted using (A) acetonitrile and (B) 10 mM ammonium acetate at pH 5.0 as mobile phases, according to the following gradient: 0 min, 3% B; 10 min, 3% B; 30 min, 10% B; 50 min, 15% B; 60 min, 20% B; 90 min, 40% B. The injection volume was 15 µL and the flow rate was set at 100 µL min^{-1}.

On the other hand, the common analytical conditions for each column used in the ^{2}D were the following:

i) Ascentis Express C_{18} (50 × 4.6 mm, 2.7 µm, Supelco, Bellefonte, CA) partially porous column using (A) water (0.1% formic acid) and (B) acetonitrile as mobile phases, eluted at 3 mL min^{-1} using two segment gradients: from 0 min to 23.4 min the ^{2}D gradient elution was 0 min, 0% B, 0.1 min, 5% B; 0.5 min, 35% B; 0.9 min, 70% B; 1 min, 90% B; 1.01 min, 0% B; 1.3 min, 0% B; from 23.4 to 80 min the employed gradient was programmed as 0 min, 0% B; 0.1 min, 5% B; 0.3 min, 35% B; 0.5 min, 40% B; 0.9 min, 50% B; 1 min, 90% B; 1.01 min, 0% B; 1.3 min, 0% B.

ii) Ascentis Express C_{18} (30 × 4.6 mm, 2.7 µm, Supelco, Bellefonte, CA) partially porous column using (A) water (0.1% formic acid) and (B) acetonitrile as mobile phases, eluted at 2 mL min^{-1}. Different gradients were applied depending on the modulation time applied. The different step gradients are detailed in Table S1.

When indicated, sets of trapping columns formed by two identical cartridges were employed including C_{18} and phenyl-hexyl (10 × 3 mm, 2.6 µm, Accucore, Thermo Scientific, Waltham,MA) stationary phases.

UV-Vis spectra were collected in the range of 190-550 nm using a sampling rate of 20 Hz, while 254, 280 and 330 nm signals were also independently recorded.

2.4. Calculations.

2.4.1 Peak capacity.

Individual peak capacity for each dimension (n_c) was calculated according to eq. 1:

$$n_c = 1 + \frac{t_G}{\overline{w}} \qquad (1)$$

where t_G is the gradient time and $\overline{w}$ is the average peak width. For ^{1}D peak capacity calculations, the average peak width was obtained from *ca.* 10 representative peaks selected along the analysis. Likewise, for ^{2}D peak capacity, as much as possible peaks were considered (*ca.* 20 peaks, depending on the analysis). Additionally, ^1n_c was also calculated considering the peak broadening factor <β>, giving rise to a corrected ^{1}D peak capacity (eq. 2), that considers the influence of the deleterious effect of undersampling. To estimate <β>, the sampling time (t_s) as well as the average width of ^{1}D peaks as standard deviation in time units ($^1\sigma$) before modulation were considered:

$$^1n_{c,corrected} = \frac{^1n_c}{\sqrt{1+0.21\left(\frac{t_S}{^1\sigma}\right)^2}} \qquad (2)$$

For each two-dimensional set-up, different peak capacity values were estimated. First of all, theoretical peak capacity was obtained following the so-called product rule, using eq. 3, considering the individual peak capacities obtained in each dimension:

$$^{2D}n_{c,theoretical} = {}^1n_c \times {}^2n_c \qquad (3)$$

As eq. 3 does not take into consideration the deleterious effects due to the modulation process as well as possible undersampling, a more realistic peak capacity value was obtained from the equation proposed by Li et al. [16] denominated here as practical peak capacity (eq. 4):

$$^{2D}n_{c,practical} = \frac{^{1}n_c \times {}^{2}n_c}{\sqrt{1+3.35\times\left(\frac{^{2}t_c\ ^{1}n_c}{^{1}t_G}\right)^2}} \quad (4)$$

being $^{2}t_c$, the ^{2}D separation cycle time, which is equal to the modulation time. This latter equation also includes the <β> parameter accounting for undersampling. Moreover, to more precisely compare among set-ups and in order to evaluate possible peak clusters along the 2D analysis and, thus, to estimate 2D space coverage, the orthogonality degree (A_O) was considered to offer the denominated 2D corrected (also known as effective) peak capacity, as follows:

$$^{2D}n_{c,corrected} = {}^{2D}n_{c,practical} \times A_O \quad (5)$$

2.4.2 Orthogonality.

Among the different approaches that have been described and published to quantify the orthogonality degree of a two-dimensional set-up [17], the method proposed by Camenzuli and Schoenmakers [18] was employed in the present work to calculate system orthogonality (A_O). This procedure takes into account the spread of each peak along the four imaginary lines that cross the 2D space forming an asterisk, that is Z_1, Z_2 (vertical and horizontal lines) and Z_-, Z_+ (diagonal lines of the asterisk). Z parameters describe the use of the separation space with respect to the corresponding Z line, allowing to semi-quantitatively diagnose areas of the separation space where sample components are clustered, thus, reducing in practice orthogonality. For the determination of each Z parameter, the S_{Zx} value was calculated, as the measure of spreading around the Z_x line, using the retention times of all the separated peaks in each 2D analysis.

2.4.3. Two-dimensional resolution.

242 The resolution metric for two-dimensional separations proposed by Peters et al. [19] was
243 employed to calculate a representative resolution value and the separation quality of each
244 set-up. This measure is based on the valley-to-peak ratio between two neighbor peaks. To
245 establish the valley-to-peak ratio between two peaks (peak 1 and peak 2), three maximum
246 intensities are considered: the maximum of the peak 1 (max1), the saddle point between
247 both peaks (S) and the maximum of peak 2 (max2), as well as the distances between max1
248 and S, $d_{1,S,}$ and the distance between S and max2, $d_{S,2}$.

249 $$d_{1,S} = \sqrt{\left(\Delta^1 t_{R1,S}\right)^2 + \left(\Delta^2 t_{R1,S}\right)^2} \quad (6)$$

250 $$d_{1,S} = \sqrt{\left(\Delta^1 t_{RS,2}\right)^2 + \left(\Delta^2 t_{RS,2}\right)^2} \quad (7)$$

251 where $\Delta^1 t_{R1,S}$ and $\Delta^2 t_{R1,S}$ are the differences on time between max1 and S in the ^{1}D and ^{2}D
252 and $\Delta^1 t_{RS,2}$ and $\Delta^2 t_{RS,2}$ the difference between S and max2 in both dimensions.
253 Then, the intensity g is defined in accordance with the graphic showed in Figure S1.
254 Intensity g is calculated by:

255 $$g = \frac{d_{1,S} h_{max2} + d_{S,2} h_{max1}}{d_{1,s} + d_{2,S}} \quad (8)$$

256 where h_{max1} and h_{max2} are the maximum intensities of peak 1 and peak 2, respectively.
257 The valley-to-peak ratio (V) is calculated as:

258 $$V = \frac{f}{g} = \frac{(g - h_s)}{g} \quad (9)$$

259 Finally, resolution (Rs) is estimated by the following equation:

260 $$Rs = \sqrt{-\frac{1}{2} \ln\left(\frac{1-V}{2}\right)} \quad (10)$$

261 In this work, the resolution measurement of two target critical pairs of peaks was
262 calculated in each instrumental configuration, and results obtained compared among
263 them.
264

3. RESULTS AND DISCUSSION

In our previous work, the first LC × LC application devoted to the profiling of secondary metabolites in licorice was developed [14]. Although the method was characterized by excellent separation capabilities, being possible to detect around 80 compounds from different metabolite families in just one sample, further optimization is desirable to increase sensitivity and to further improve performance. This is mainly interesting due to the fact that this sample is a very diverse and complex mixture of some closely related components, such as glycosylated flavanones and chalcones among other polyphenols as well as triterpene saponins. In the present work, we have applied several strategies, using licorice as a model complex real food sample in order to quantitatively evaluate the attainable performance by introducing new changes in the interface.

3.1. Non-focusing modulation.

3.1.1. Influence of transfer volume/fraction solvent.

The most-extended approach to interface both dimensions in LC × LC is the use of two identical sampling loops installed on the switching valve(s) acting as modulator. In our original method, two 30 μL sampling loops were employed with satisfactory results. However, modifications at the interface and columns combination levels could further improve two of the most important points in a comprehensive LC separation: ^{1}D undersampling and ^{2}D band broadening. These two parameters have a clear deleterious effect both on the resolving power as well as on the attainable peak capacity, and thus, should be minimized. The coupling between HILIC and RP is characterized by a very good degree of orthogonality, thus, being very attractive for the analysis of complex samples. Nevertheless, it generates a solvent mismatch during the transfer of ^{1}D effluent, considering that the weaker solvent in the ^{1}D is the stronger one on the ^{2}D environment.

290 According to the intensity of this issue, the resulting ^{2}D separations may be completely
291 ruined, or just worsened to a certain degree depending on the extent of the associated
292 band broadening effect. For this reason, one of the possible strategies to avoid or reduce
293 the mentioned solvent strength mismatch is to dilute the ^{1}D effluent before its transfer to
294 the ^{2}D. When using a non-focusing modulation procedure based on sampling loops, this
295 effect may be obtained through the use of loops with an internal volume higher than the
296 strictly required to collect the ^{1}D effluent during the length of a modulation. That way,
297 ^{1}D effluent supposes only part of the available loop volume whereas the rest is filled with
298 ^{2}D starting mobile phase. However, it has to be also considered that, since short columns
299 are employed in the ^{2}D to obtain fast separations, the increase on the sampling loop
300 volume, which is also the injection volume for each individual ^{2}D separation, may
301 negatively influence the separation [20].
302 Accordingly, the first step was to study the effects of sampling volume and fraction
303 solvent on the ^{2}D, comparing the separation attainable using sampling loops with different
304 internal volume, i.e., 20, 30 and 50 µL, operated in forward elution. To do that,
305 experimental conditions based on the use of the ZIC-HILIC microbore column in the ^{1}D
306 and the use of a 50 × 4.6 mm, 2.7 µm C_{18} partially porous column in the ^{2}D, using 78 s
307 as modulation and ^{2}D analysis time, were applied (see Section 2.3). As can be observed
308 in Table 1 and Figure S2A-C, the results in terms of overall separation, resolution and
309 orthogonality were fairly similar. Interestingly, a slight but noticeable increase on
310 theoretical peak capacity was obtained when the sampling loops volume was bigger. This
311 trend would correspond to a decrease on average ^{2}D peak widths as a result of higher
312 dilution of the ^{1}D effluent and, thus, to the injection of each fraction on a weaker solvent,
313 which helps to improve peak shapes with respect to less diluted fractions. As can be also
314 observed in Table 1, 50 µL fractions injected in the ^{2}D meant an injection volume of 10%

315 of column void volume, considering that partially porous particles may occupy around
316 40% of the total available column inner volume [21,22]. Thus, the reduction on the
317 fraction solvent strength obtained when using 50 µL sampling loops (a 2.6-fold dilution)
318 was able to make up for the possible deleterious effect due to increased injection volume.
319 In fact, the use of 10% column void volume was significantly higher than the 3%
320 previously reported in order to not get peak distortion [20]. In spite of the increment
321 obtained in theoretical peak capacity, no practical gains on separation were observed
322 (Figure S2A-C).
323
324 3.1.2. Influence of sampling frequency.
325 Possible enhancements on resolving power could be obtained minimizing the effect of ^{1}D
326 undersampling. One of the concepts that characterize the performance of an on-line LC ×
327 LC method is the importance of maintaining the separation obtained in the ^{1}D during the
328 transfer of ^{1}D effluent to the ^{2}D. If the sampling process is too slow to collect fractions
329 where two well separated ^{1}D peaks are involved, undersampling arises; in that case, a
330 remix of these previously separated peaks occurs in the transfer process, producing a loss
331 of the ^{1}D separation and peak capacity. To reduce this negative effect, higher sampling
332 frequencies should be applied, in order to obtain more ^{1}D fractions analyzed in the ^{2}D.
333 Murphy et al. [23] established the widely-accepted rule of sampling 3-4 times each ^{1}D
334 peak to solve the remix problem and to maintain the ^{1}D separation. However, in this case,
335 due to instrumental limitations on maximum bearable backpressure, it was not possible
336 to reduce the analysis time used with the 50 mm C_{18} partially porous column employed.
337 Changes in the ^{2}D gradient did not produce any noticeable improvement either. For this
338 reason, an even shorter column was tested. A 30 × 4.6 mm C_{18} partially porous column
339 (2.7 µm) was coupled to the formerly optimized ^{1}D. By using this shorter column, a

340 proper separation was obtained allowing a decrease on total ^{2}D analysis time (gradient
341 time + re-equilibration time) to just 60 s. Under these analytical conditions, the use of the
342 three different transfer volumes was studied (Figure S2D-F). As can be observed from
343 the data summarized in Table 1, theoretical peak capacities obtained using the 30 mm
344 column were lower than those attainable using the 50 mm, as a result of the great
345 dependence of $^{2}n_{c}$ on the available gradient time. However, as a result of the faster ^{1}D
346 sampling rate applied when the shorter column was used, both orthogonality and
347 resolution of pair 1 were improved, independently of the transfer volume employed (see
348 Table 1). This improvement was more pronounced when using 50 µL sampling loops, as
349 deduced from the data shown on Table 1 and illustrated in Figure 1A-B and Figure S2. In
350 this latter set-up, the ^{2}D injection volume was equal to 17% of column void volume.
351 Although this relative injection volume is rather high, no appreciable distorted peaks were
352 detected compared to 20 and 30 µL transfer volumes; indeed, the dilution effect achieved
353 using 50 µL, again allowed better retention of compounds due to the greater dilution in
354 ^{2}D compatible mobile phase could produce a better interaction of the analytes with the
355 stationary phase (see Figure S2D-F and Figure S3).
356 Although these conditions clearly improved the results attainable using the longer
357 column, the use of higher separation temperature was also explored to investigate if
358 proper ^{2}D separations could be obtained in even shorter analysis times, thus, further
359 increasing ^{1}D sampling rate. To do that, the ^{2}D column was thermostated at 40 ºC and
360 several changes were applied to the gradient profile to adapt the separation to a total 39
361 and 50 s analysis times (Table S1). In order to establish a wider evaluation, the results
362 obtained using the different mentioned modulation times (39, 50 and 60 s) were also
363 compared with the longer 78 s ^{2}D modulation time previously employed with the 50 mm
364 column. In this regard, considering that faster ^{1}D sampling rates imply that less ^{1}D

effluent volume is transferred to the ^{2}D, the use of sampling loops volume of 50 µL was considered too high; for this reason, to perform these series of experiments, 20 µL sampling loops were installed in the switching valve, allowing more discrete transfers equivalent to 7% of total ^{2}D column void volume. Results are summarized in Table S2 and Figure 2. As can be observed, as the modulation time was reduced, $^{2}n_c$ values also decreased, as a result of the great influence that this value retains from the available ^{2}D t_G. In consequence, the practical 2D peak capacity also tended to decrease. However, the observed decrease is not more pronounced thanks to the better peak shapes obtained as a result of a more pronounced gradient slope and higher dilution effect when using 39 s as modulation time; at those conditions, just 9.5 µL of ^{1}D effluent were transferred in each modulation, whereas the rest of the sampling volume was filled with ^{2}D mobile phase, thus, helping to reduce the solvent strength mismatch. Moreover, the effect of higher sampling rate is also illustrated on the attainable resolution between the two pairs of compounds studied. As illustrated in Figure 2, resolution between pair 1 improved when reducing the modulation time. In addition, compounds in pair 2 remained coeluted using modulation times of 78 and 60 s, but they could be separated using shorter modulation times.

3.2. Focusing modulation using trapping columns.

One of the possible implementations to reduce ^{2}D band broadening and to increase sensitivity limiting dilution is the use of trapping columns in the valve-based modulator. Reduction on band broadening is accomplished by introducing a focusing effect, considering that the analytes eluting from the ^{1}D would be entrapped in the trapping column during the collection position. Once the valve is actuated, ^{2}D mobile phase would desorb the analytes in discrete bands, injecting them into the ^{2}D column. Even if this

390 approach has a good potential, its use is very limited compared to regular loops-based
391 modulation. In the food analysis field, only C_{18} trapping columns have been reported
392 [10,24], in order to exactly match the selectivity of the ^{2}D column. In the present work,
393 the use of trapping columns-based modulation to increase resolving power and sensitivity
394 is extended to other stationary phases. Namely, the use of C_{18} and phenyl-hexyl trapping
395 columns have been explored. The traps (10 × 3.0 mm, 2.6 µm) were installed in the
396 modulator using the minimum possible extra volume for connections. The trapping
397 columns void volume was 42 µL. Moreover, two elution configurations were compared,
398 namely, forward and backflush elution. The use of the shortest available ^{2}D column was
399 maintained, setting a modulation time of 60 s. Table 2 reports the most important method
400 parameters related to these analyses. As can be observed, very similar results could be
401 obtained using the two stationary phases available as well as both elution modes in terms
402 of peak capacity and orthogonality attainable. Interestingly, using both elution modes
403 resolution of critical pair 1 was maintained with respect to the best value attainable using
404 non-focusing modulation, whereas, pair 2, that coeluted using the same separation
405 conditions (60 s modulation time) with sampling loops, was also resolved. In any case,
406 forward elution produced better resolution results for both tested stationary phases.
407 Moreover, as can be appreciated from Figure S4, in general, ^{2}D peak shapes were
408 improved also under forward elution compared to backflush elution. This effect would be
409 obtained as a result of the longer available interaction allowed under forward elution,
410 bearing in mind that the trapping column was not fully filled with ^{1}D effluent during the
411 collection position (see Figure 3A,B). In addition, although reduced to a minimum, a 2
412 µL tube was necessary to connect the trapping columns to the valve; consequently, there
413 was a small fraction of ^{1}D effluent that did not enter the trapping column when backflush
414 elution was employed (Figure 3B).

3.3. Focusing using active modulation.

The use of active modulation is a further evolution of the direct use of trapping columns. This modulation procedure, recently proposed [13], is based on the use of an additional make-up flow of a weak solvent for the ^{2}D in order to reduce the strength of the ^{1}D effluent prior entering the trapping column. This way, the interaction between the analytes and the functional groups in the trap is fostered, as illustrated in Figure 3C. Therefore, considering the high potential and relative simplicity that this implementation may have, it is worth to study its application to complex food samples. Considering the ^{1}D and ^{2}D mobile phases compositions, it was decided to use ultrapure water (0.1% formic acid) as make-up flow. It has been previously observed that a flow rate for the additional make-up flow 7-times higher than the ^{1}D flow rate was appropriate to achieve the desired effect [13]. However, to further study the possible influence of make-up flow rate on the overall separation performance, three different flow rates for each set of trapping columns (C_{18} and phenyl-hexyl) were tested, i.e., 5-, 7- and 9-times the ^{1}D flow rate. Table 3 summarizes the data describing the performance attained using active modulation for the profiling of secondary metabolites in licorice. As can be appreciated, as for trapping columns, the performance attainable using both stationary phases was very similar. In both cases, the use of higher make-up flow rates allowed a slight improvement on peak capacity, whereas orthogonality values were essentially maintained. More relevant was the improvement observed for the resolution between the two studied pairs; in this regard, the use of make-up flow rates 9-times higher than the ^{1}D flow rate produced the best results (Figure 4).

3.4. Overall comparison.

As already described in the previous sections, different approaches have been considered to further improve the separation capabilities of the initial HILIC × RP method directed towards the profiling of secondary metabolites in licorice. In general, the use of focusing modulation, either using trapping columns or active modulation, allowed a clear improvement on the separation of the complex metabolite profile of this sample (Figure 1). In fact, these two approaches allowed obtaining good degrees of resolution between the studied pairs (Figures 4 and S4). In general, better separations were obtained using trapping columns in forward elution mode and using active modulation with make-up flow rates 9-times the ^{1}D flow rate. Although in both cases, the two stationary phases studied produced comparable performance, the phenyl-hexyl particles were slightly better than C_{18} particles. Under these conditions, similar orthogonality values as well as resolution between the critical pairs were obtained (Tables 2 and 3). However, practical peak capacities were significantly higher using active modulation (2131 vs 1811), and thus, this procedure resulted more favorable. The use of non-focusing modulation by sampling loops could only provide comparable performance in some aspects when modulation time was significantly reduced, thus, increasing ^{1}D sampling rate. However, due to very fast ^{2}D separations, the total 2D peak capacity attainable was severely compromised with respect to active modulation.

With the aim to further obtain more data illustrating the performance of each procedure, the attainable sensitivity under each separation conditions was studied by analyzing peak S (Figure 5). Values of normalized sensitivity for each set-up are included in Tables 1-3. This value was obtained by considering the sensitivity for peak S in the original method with respect to the sensitivity obtained in each case. As can be observed from those results, the set-up involving the use of active modulation using phenyl-hexyl traps and make-up flow at 9-time ^{1}D flow rate produced the highest sensitivity enhancement.

Consequently, active modulation was shown again as the best possible alternative set-up for the profiling of secondary metabolites in licorice by HILIC × RP in order to further enhance both resolving power and sensitivity.

In this regard, theoretically, further sensitivity gains could be obtained if a column with higher sample loadability is used in ^{1}D. For this reason, a last attempt was made using the optimum separation conditions but increasing the ^{1}D column internal diameter to 2.1 mm. That column allowed an increase on the injection volume to 15 µL, although higher ^{1}D flow rates were also needed to maintain the ^{1}D separation. This would have a deleterious effect on the fraction volume transferred to ^{2}D, but considering that active modulation was employed with trapping columns, the fraction volume should not have such a critical influence on the coupling. As shown in Table S3, the normalized sensitivity obtained was further increased with respect to the use of the microbore ^{1}D column (Figure 5F); however, the separation obtained was severely hampered, and the resolution between the critical pairs studied was completely lost (Table S3). Thus, this modification was not considered favorable, bearing in mind that compromises should be always taken between sensitivity, resolving power and overall peak capacity obtainable.

4. CONCLUSIONS.

In the present contribution, different strategies are proposed and compared to further increase HILIC × RP method performance for the analysis of complex food samples, using licorice as a model sample. When using non-focusing modulation based on sampling loops, the use of very short columns (30 mm) in the ^{2}D was shown to be beneficial to increase performance, taking advantage of higher sampling loops volume to increase solvent dilution, thus, minimizing the deleterious effects due to solvent strength

490 mismatch between HILIC and RP. However, the use of focusing modulation procedures
491 was demonstrated to be able to increase not only resolving power but also peak capacity,
492 reducing the effects of solvent mismatch at the same time that producing a focusing effect
493 at the beginning of the ^{2}D analyses. In addition, significant sensitivity gains could be also
494 obtained through the use of active modulation with a relatively high make-up flow rate.
495 A total of 94 peaks were successfully separated in the set-up involving the use of active
496 modulation with phenyl-hexyl trapping columns and a make-up flow rate 9-times higher
497 than the corresponding ^{1}D flow rate, compared to the initial method (79 peaks), increasing
498 sensitivity more than twice. In summary, the results obtained demonstrate the great
499 applicability and potential that active modulation may have for the profiling of complex
500 food samples by HILIC × RP.
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502
503 **ACKNOWLEDGEMENTS.**
504 The authors would like to thank Project AGL2014-53609-P (MINECO, Spain) for
505 financial support.

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580
581

582 **FIGURE LEGENDS**

583 **Figure 1.** Resolution obtained for peaks included in critical pair 1 (white oval) and in
584 critical pair 2 (black oval) in the different set-ups studied. A, using 50 µL sampling loops
585 in combination with a 50 mm long column in the ^{2}D; B, using 50 µL sampling loops in
586 combination with a 30 mm long column in the ^{2}D; C, using Phenyl-hexyl trapping
587 columns with forward elution, and; D, using active modulation with phenyl-hexyl traps
588 and make-up flow rate equal to 9-times ^{1}D flow rate.

589

590 **Figure 2.** Dependence of practical peak capacity ($^{2D}n_{c,practical}$) (●), 2D resolution reached
591 for pair 1 (■), and 2D resolution for pair 2 (×) on modulation time. For detailed separation
592 conditions, see section 2.3.

593

594 **Figure 3.** Hypothetical scheme of the retention/elution of secondary metabolites from
595 licorice into the trapping columns under forward elution mode (A), backflush elution
596 mode (B), and active modulation (C) set-ups studied, following the procedure: 1)
597 Trapping column filled with ^{2}D initial mobile phase (injection position, just after valve
598 actuation); 2) Trapping column filled with ^{1}D effluent fraction (collection position)
599 diluted in strong (A and B) or weak (C) solvent; 3) start of ^{2}D gradient. Arrows indicate
600 flow direction.

601

602 **Figure 4**. Resolution obtained by using active modulation with phenyl-hexyl trapping
603 columns of the two pairs of the studied peaks, using make-up flow rates equal to 5- (A),
604 7- (B) and 9-times (C) ^{1}D flow rate, and practical scheme of the calculation of the valley-
605 to-peak ratio used for the estimation of resolution between critical pairs 1 and 2 (D).

606

607 **Figure 5.** Panel A: 2D-plot (254 nm) of the separation obtained in the original method.
608 Sensitivity comparison (peak S) of the original method with the set-up of each modulation
609 configuration using: B, 50 µL sampling loops in combination with 50 mm length column
610 in the ^{2}D; C, 50 µL sampling loops in combination with 30 mm length column in the ^{2}D;
611 D, C_{18} trapping columns with forward elution; E, active modulation with phenyl-hexyl
612 traps and make-up flow rate equal to 9-times ^{1}D flow rate, and; F, sensitivity gain with
613 the 250 × 2,1 mm, 3,5 µm ^{1}D column. (Retention times of analyses with different ^{2}D
614 gradient are aligned to help the comparison).

615 **Table 1.** Comprehensive two-dimensional method parameters applied to the profiling of secondary metabolites from licorice using non-focusing
616 modulation

		^{2}D - C_{18} 50 × 4.6 mm, 2.7 µm			^{2}D - C_{18} 30 × 4.6 mm, 2.7 µm		
	Sampling loop volume	20 µL	30 µL	50 µL	20 µL	30 µL	50 µL
^{1}D	L (mm)	150	150	150	150	150	150
	I.D. (mm)	1.0	1.0	1.0	1.0	1.0	1.0
	Particle size (µm)	3.5	3.5	3.5	3.5	3.5	3.5
	Flow rate (µL min^{-1})	15	15	15	15	15	15
	$\overline{w}$ (min)	2.69	2.69	2.69	2.47	2.47	2.47
	$^{1}n_c$	30	30	30	33	33	33
	<β>	1.33	1.33	1.33	1.25	1.25	1.25
	$^{1}n_c$ corr.	23	23	23	27	27	27
^{2}D	$\overline{w}$ (s)	1.06	0.90	0.78	1.02	1.00	1.00
	$^{2}n_c$	75	88	101	60	61	61
LC × LC	Analysis time (min)	80	80	80	80	80	80
	t_s	1.93σ	1.93σ	1.93σ	1.62σ	1.62σ	1.62σ
	Modulation time (min)	1.3	1.3	1.3	1.0	1.0	1.0
	M – number of modulations	62	62	62	80	80	80
	$^{2}V_{inj}$ (V dilution)	20 µL (0.5 µL)	30 µL (10.5 µL)	50 µL (30.5 µL)	20 µL (5.0 µL)	30 µL (15.0 µL)	50 µL (35.0 µL)
	% ^{2}D column void volume	4%	6%	10%	7%	10%	17%
	Gradient delay volume (mL)	0.72	0.74	0.76	0.48	0.48	0.51
	^{2}D column operation preassure (bar)	299	295	298	187	185	190
	Z_1	0,84	0,91	0,92	0,89	0,82	0,85
	Z_2	0,97	0,96	0,99	0,97	0,98	0,97
	Z_-	0,69	0,77	0,81	0,91	0,89	0,86
	Z_+	0,83	0,87	0,84	0,99	0,95	0,99
	A_O	68%	76%	79%	82%	82%	84%
	Resolution pair 1	0.65	0.67	0.70	0.75	0.83	0.89
	Resolution pair 2	-	-	-	-	-	-
	Normalized sensitivity	0.85	1.00	1.37	1.08	1.32	1.61
	$^{2D}n_c$ theoretical	2250	2640	3030	1980	2013	2013
	$^{2D}n_c$ practical	1730	1964	2253	1706	1736	1780
	$^{2D}n_c$ corr.	1401	1493	1780	1399	1424	1495

617
618 <β>, average ^{1}D broadening factor; $^{1}n_c$ corr.: calculated according to eq. 2; t_s, sampling time; A_O, orthogonality; $^{2D}n_c$ theoretical: $^{1}n_c \times {}^{2}n_c$; $^{2D}n_c$ practical: calculated according to
619 eq. 4; $^{2D}n_c$ corr.: $^{2D}n_c \times A_0$

620 **Table 2.** Comprehensive two-dimensional method parameters applied to the profiling of

621 secondary metabolites from licorice using trapping columns-based focusing modulation.

		Forward elution		Backflush elution	
	Trapping column	C18	Phenyl-hexyl	C18	Phenyl-hexyl
^{1}D	L (mm)	150	150	150	150
	I.D. (mm)	1.0	1.0	1.0	1.0
	Particle size (µm)	3.5	3.5	3.5	3.5
	Flow rate (µLmin^{-1})	15	15	15	15
	$\overline{w}$ (min)	2.11	2.11	2.11	2.11
	$^{1}n_c$	39	39	39	39
	<β>	1.32	1.32	1.32	1.32
	$^{1}n_c$ corr.	30	30	30	30
^{2}D	$\overline{w}$ (s)	0.97	0.96	0.98	0.98
	$^{2}n_c$	63	64	62	62
LC × LC	Analysis time (min)	80	80	80	80
	t_s	1.88σ	1.88σ	1.88σ	1.88 σ
	Modulation time (min)	1.0	1.0	1.0	1.0
	M – number of modulations	80	80	80	80
	Gradient delay volume (mL)	0.51	0.51	0.44	0.44
	^{2}D column operation preassure (bar)	260	258	253	254
	Z_1	0,94	0,86	0,83	0,87
	Z_2	0,92	0,94	0,97	0,93
	Z_-	0,94	0,89	0,92	0,97
	Z_+	0,93	0,98	0,92	0,83
	A_0	87%	84%	83%	81%
	Resolution pair 1	0.80	0.80	0.79	0.81
	Resolution pair 2	0.81	0.85	0.72	0.78
	Normalized sensitivity	1.15	0.99	0.59	0.75
	$^{2D}n_c$ theoretical	2457	2496	2418	2418
	$^{2D}n_c$ practical	1792	1811	1777	1777
	$^{2D}n_c$ corr.	1559	1521	1475	1439

622 <β>, average ^{1}D broadening factor; $^{1}n_c$ corr.: calculated according to eq. 2; t_s, sampling time; A_O,
623 orthogonality; $^{2D}n_c$ theoretical: $^{1}n_c \times {}^{2}n_c$; $^{2D}n_c$ practical: calculated according to eq. 4; $^{2D}n_c$ corr.: $^{2D}n_c \times A_O$
624

Table 3. Instrumental parameters employed and method performance descriptors from the use of active modulation for the profiling of secondary metabolites from licorice.

		C18 trapping columns			Phenyl-hexyl trapping columns		
	Make-up flow rate	5 × ^{1}F	7 × ^{1}F	9 × ^{1}F	5 × ^{1}F	7 × ^{1}F	9 × ^{1}F
^{1}D	L (mm)	150	150	150	150	150	150
	I.D. (mm)	1.0	1.0	1.0	1.0	1.0	1.0
	Particle size (µm)	3.5	3.5	3.5	3.5	3.5	3.5
	^{1}F (Flow rate, µL min^{-1})	15	15	15	15	15	15
	$\overline{w}$ (min)	2.11	2.11	2.11	2.11	2.11	2.11
	$^{1}n_c$	39	39	39	39	39	39
	<β>	1.33	1.33	1.33	1.32	1.32	1.32
	$^{1}n_c$ corr.	1.32	1.32	1.32	1.32	1.32	1.32
^{2}D	$\overline{w}$ (s)	0.93	0.84	0.81	0.96	0.84	0.81
	$^{2}n_c$	66	73	75	63	73	75
LC × LC	Analysis time (min)	80	80	80	80	80	80
	t_s	1.88σ	1.88σ	1.88σ	1.88σ	1.88σ	1.88σ
	Modulation time (min)	1.0	1.0	1.0	1.0	1.0	1.0
	M – number of modulations	80	80	80	80	80	80
	Gradient delay volume (mL)	0.49	0.49	0.49	0.49	0.49	0.49
	Column operation preassure (bar)	266	267	266	259	261	262
	Z_1	0,87	0,89	0,90	0,87	0,87	0,88
	Z_2	0,98	0,96	0,96	0,96	0,94	0,94
	Z_-	0,91	0,92	0,91	0,87	0,89	0,89
	Z_+	0,93	0,93	0,94	0,96	0,97	0,95
	A_O	85%	86%	86%	84%	84%	84%
	Resolution pair 1	0.69	0.82	0.85	0.71	0.91	0.93
	Resolution pair 2	0.81	0.86	0.88	0.82	0.86	0.88
	Normalized sensitivity	1.46	1.59	1.98	0.91	1.67	2.01
	$^{2D}n_c$ theoretical	2574	2847	2925	2457	2847	2925
	$^{2D}n_c$ practical	1888	2075	2128	1806	2070	2131

$^{2D}n_c$ corr.	1605	1785	1830	1517	1739	1790

<β>, average ^{1}D broadening factor; $^{1}n_c$ corr.: calculated according to eq. 2; t_s, sampling time; A_O, orthogonality; $^{2D}n_c$ theoretical: $^{1}n_c \times {}^{2}n_c$; $^{2D}n_c$ practical: calculated according to eq. 4; $^{2D}n_c$ corr.: $^{2D}n_c \times A_O$

629