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Journal of Chromatography A - 2019, Vol. 1614, No. 460729, 2020.

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Chiral x Achiral Multidimensional Liquid Chromatography. Application to the enantioseparation of dintitrophenyl amino acids in honey samples and their fingerprint classification

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PII: S0021-9673(19)31161-6
DOI: https://doi.org/10.1016/j.chroma.2019.460729
Reference: CHROMA 460729

To appear in: *Journal of Chromatography A*

Received date: 21 September 2019
Revised date: 15 November 2019
Accepted date: 20 November 2019

Please cite this article as: A. Acquaviva , G. Siano , P. Quintas , M.R. Filgueira , C.B. Castells , Chiral x Achiral Multidimensional Liquid Chromatography. Application to the enantioseparation of dintitrophenyl amino acids in honey samples and their fingerprint classification, *Journal of Chromatography A* (2019), doi: https://doi.org/10.1016/j.chroma.2019.460729

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Highlights

- Novel LCxLC configuration for enantioresolution of amino acids
- Chiral x achiral 2DLC is implemented to determine amino acids profiles
- Chiral Quinine phase and C18 are combined to increase separation space.
- Splitting the ^{1}D eluent by an active splitter pump improves the separation quality
- Amino acids from honey samples from different origins were determined

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Chiral x Achiral Multidimensional Liquid Chromatography. Application to the enantioseparation of dintitrophenyl amino acids in honey samples and their fingerprint classification

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Abstract

Most HPLC enantiomer separations are performed with columns packed with a chiral stationary phase (CSP) operated with an achiral mobile phase. The intrinsically limited chemical selectivity of most CSPs to the simultaneous resolution of several pairs of enantiomers means that complex mixtures of diverse pairs of enantiomers cannot be resolved in a single run due to peak overlapping. Moreover, some drawbacks remain when the analyte is present in very complex samples containing other achiral compounds which can co-elute with the enantiomer peaks. Multidimensional chromatography becomes an option to increase peak capacity and resolve these samples.

The aim of this work was to study an online fully comprehensive 2D-LC mode utilizing a combination of a chiral column in the first dimension and an achiral column in the second dimension. The 2D-LC system was built with an active flow splitter pump in order to easily adjust the volume of sample transferred into the second dimension and to independently optimize the flow rate in the first dimension.

The present LCxLC method was optimized for the separation of amino acids present in honey samples, taking into account key parameters that influence the bidimensional peak capacity (orthogonality, sampling frequency, etc.). The amino acids have been preconcentrated on a cation-exchange column followed by derivatization. Several amino acids present in different honey samples have been identified and the data generated has been analyzed by principal component analysis.

Keywords

Comprehensive 2D-LC; ChiralxAchiral method; Active splitter pump; Honey samples; D,L-amino acids; Principal Component Analysis

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1. Introduction

The determination of α-amino acid enantiomers in biological, environmental, and food samples is a widely investigated topic in analytical chemistry. All proteinogenic amino acids, with the exception of glycine, are chiral and their presence in foodstuffs, beverages, and health products are associated with one or more properties of those products [1–3].

A great variety of analytical methods for the analysis of amino acids based on GC, HPLC and HPCE have been reported [4–7]. Most of these methods have been established for the simple and rapid determination of a number of amino acid enantiomers in diverse samples. However, the determination of small amounts of D-amino acids in an enantiomeric mixture in addition to the complexity of the sample matrices has always been a challenge in method development. Common interferences are peptides, other amino acids, and amino compounds. Thus, highly selective analytical methods are essential for the research involved in the identification, classification, studies about biological effects, and quality control of several processed food.

In this context, there is no doubt that two-dimensional liquid chromatography (2D-LC) is a powerful tool in the separation and analysis of complex samples [8,9]. By combining different separation modes, this technique can provide enough selectivity and the peak capacity needed for such challenging separations. When comparing with off-line 2D-LC, a comprehensive on-line 2D-LC system offers the advantage of the automation for transferring the samples from the first dimension into the second dimension, repeatability, relatively short analysis times, while retaining the high peak capacity. This mode of 2D-LC requires very fast separation in the second dimension and the compatibility between mobile phases used in both first and second dimensions. On the other hand, off-line 2D-LC systems have the advantage of providing higher peak capacity, but at the expense of a large amount of labor and longer total analysis time.

Online 2D-LC approaches have also been proposed for enantiomeric separations. In most studies reporting chiral separations, the achiral separation system is used in the first dimension (^{1}D) whereas the second dimension (^{2}D) separations are conducted mainly in heart-cut mode. Such configuration is founded on the relatively slow speed of chiral separations, i.e. usually slow kinetics and columns of lower efficiency when compared to more common achiral LC columns of the same geometrical features. Consequently, the use of chiral separations on the second dimension in fully comprehensive 2D-LC experiments is scarce and limited to readily resolved enantiomeric mixtures [10–12]. An alternative method reported in the literature has been the use of supercritical fluid chromatography (SFC) in the second dimension in order to speed up chiral separations [13–15]. Iguiniz and co-workers used the quiral column in the second dimension through an SFC system by selective LCxLC (sLCxLC) [16]. This alternative has the disadvantage of solvent compatibilities. Another approach reported consisted in using peak storage in the sample loops of the interface between the two dimensions for subsequent analysis (in heart cut or multiple heart cut modes)[17,18]. In this case but the analysis time and technical complexity are the main flaws for those ones, particularly when several “heart-cuts” must be separated on a second chiral column. Furthermore, in most of the enantioselective cases of multiple heart cut 2D-LC analysis of

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amino acids, only a subset of targeted amino acids of particular interest has been analyzed [19,20]. Wang and co-workers used off-line LC×LC separations to characterize the enantiomeric composition of free amino acids in tea in another example of non-comprehensive 2D-LC. Amino acids were derivatized with 9-fluorenylmethoxycarbonyl (FMOC) chloride prior to 2D-LC using the RP mode in the first dimension followed by the chiral mode in the second dimension[21].

The objective of the present work was to demonstrate the possibility of setting the relatively slow chiral separation in the first dimension whereas the achiral separation is set at the high velocities required for the second dimension in comprehensive LCxLC. The chiral column consists in a stationary phase based on quinine linked to mercaptopropylsilica. This column has been previously used to enantioseparate dinitrophenyl-amino acids under anion exchange mode [22]. The 2D-LC system is completed with a very fast second dimension by using a short column of reduced particle diameter operated at relatively high flow-rate to minimize under sampling. The targeted field of application was the determination of enantiomeric amino acid profiles in honey samples collected from different regions of Mendoza province in Argentina.

Since comprehensive two-dimensional chromatography (LCxLC or GCxGC) can generate a very large amount of multidimensional data, the chemometric analysis of these data requires strategies to reduce the multiple dimensions, implying the interpretation and highlighting the chemical differences between samples. For this purpose Principal Component Analysis (PCA) has been successfully applied to this type of data [23–25]. As it has been reported [23], it is computationally useful to reduce the data trough peak-picking approaches, preserving fundamental information of the peaks (areas and retention times) to maintain the unique characteristics of the different samples. Since the number of peaks found in each sample can be different, it is necessary to standardize this value among all samples through the binning of retention times. This, in turn, has the advantage of diminishing the effect of peak shifting in both chromatographic dimensions [23]. In the present report, both peak counting and retention times binning were implemented before PCA. Additionally, after PCA, the analysis of the loadings of some principal components was carried out through a "bubble plot"-like methodology.

2. Experimental

2.1. Reagents and Materials

Unless otherwise stated, all chemicals employed were reagent grade. 1-fluoro-2,4-dinitrobenzene (DNPF) and formic acid (85 % w/w) were obtained from Fluka (Buchs, Switzerland). The amino acid standards arginine (Arg), asparagine (Asn), proline (Pro), ornithine (Orn), isoleucine (Ile), leucine (Leu), serine (Ser), threonine (Thr), valine (Val), phenylalanine (Phe), alanine (Ala), lysine (Lys), tryptophane (Trp), tyrosine (Tyr), methionine (Met), norleucine (Nor), glycine (Gly), 2-aminon-n-butyric acid (AB), histidine (His) and tert-leucine (Tert) were purchased from BDH (Poole, UK); hydrochloric acid from Merck (Darmstadt, Germany);

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ammonium hydroxide (28% w/w), borax, potassium chloride and isopropyl alcohol from Anedra (Argentina) and HPLC-grade acetonitrile (ACN) and methanol (MeOH) were from Baker (Mexico City, Mexico). Water was purified by means of a Milli-Q Purification System (Simplicity, Millipore, MA, USA).

2.2. Standard solutions

Standard solutions of individual amino acids (AAs) were prepared in 0.1 M HCl, stored at 4 °C and diluted before using. Borate buffer was prepared from 0.2 M borax, and adjusted to pH 9.20 (final concentration: 0.4 M buffer solution). The stock solution of 1-fluoro-2,4-dinitrobenzene (DNPF) was prepared by dissolving the solid in ACN to obtain a 0.032M solution. A 0.1M HCl solution was prepared to stop the derivatization reaction and to eliminate the 2,4-dinitrophenolate that would interfere in the chromatogram.

2.3. Amino acid derivatization

For a sensitive determination, amino acids were derivatized by Sanger's reaction with 1-fluoro-2,4-dinitrobenzene to form dinitrophenyl amino acids. Derivatives gain a suitable chromophore group for sensitive detection at 365 nm. Most of the amino acids selected in this work (seventeen) have only one reactive amino group to be DNP-labeled, but Orn, Tyr and Lys have two reactive groups.

The derivatization reaction was carried out by mixing 185 µL of borate buffer 50 mM solution at pH 9.20, 185 µL of amino acid standard solution or 185 µL of the sample after treatment, and 70 µL of DNPF 0.032 M solution. The mixture was heated in a water bath at 60 °C for 40 min, cooled down and finally the solution was acidified with 50 µL of 0.1 M HCl. A reaction blank was also prepared following the same procedure. All final solutions were filtered through a 0.22 µm Nylon membranes (Micron Separations, Inc., Westborough, MA, USA).

2.4. Honey sample preparation

1.0 gram of honey was dissolved in 5.00 mL of 0.01 M HCl. The amino acids were retained on a cation exchange resin (Dowex 50W). The columns were washed with pure water and the amino acids were eluted using 8 M NH_4OH (twice) and finally (50:50) water: MeOH as elution solvent. The effluent was evaporated to dryness and reconstituted in 200 µL of water prior to derivatization.

2.5. Instrumentation

Fully comprehensive online 2D-LC experiments were performed using an Agilent system (Agilent Technologies Waldbronn, Germany) assembled with the following components. First dimension: 1260 quaternary pump, 1260 autosampler, 1100 column compartment, and 1200 DAD detector mounted with a 1-µL flow cell and a pressure relief device between the DAD detector and the interface. The interface consisted of a 2-position/8 port valve equipped with two 20-µL sampling loops. Second dimension: a 1290 binary high-pressure gradient UHPLC

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pump, 1100 column compartment, and a 1290 DAD detector also equipped with a 1-µL flow cell. The signals were acquired at 365 nm at 80 Hz frequency. A binary pump (1290) was used as the active flow splitter pump to independently control the volumes transferred into the ^{2}D. The instrument was controlled with OpenLab CDS. A schematic diagram of the system is shown in Figure 1.

2.6. Chromatographic conditions

Three columns were used in this work. The chiral stationary phase, developed previously in our research group, consisted of quinine molecule linked to 5-µm particle size silica through mercaptopropyl bridge groups followed by an endcapping reaction with 1-hexene (CSP-QN-EC) [22]. The CSP was packed into a100 x 2.1 mm stainless steel hardware. The optimum column temperature was 30 °C. Different ^{1}D gradients were evaluated with the optimum being: 0 (10min) – 100 % B in 50 min. A: 50 mM ammonium formate pH 6.30 and B: 50:50 ACN:MeOH. Flow rate was kept at 0.3 mL/min.

The second dimension columns employed were either C18 or phenyl-butyl (3.3 x 2.1 mm and 3 µm particle diameter) Triart series from YMC (Kyoto, Japan). Column temperature was 65°C, mobile phase gradient was 0-70 %B in 0.25 min, mobile phase A: 50 mM formic acid and B: ACN. Flow rate was 3 mL/min. For the interface between both dimensions the sampling time was 0.3 min, and the 20 µL loops were filled to approximately 40% by adjusting the active splitting pump flow rate.

2.7. Data Processing

The data were acquired by the OpenLab CDS software as a single run for each 2D experiment; the data were then exported and processed using Matlab (R2012b, The Mathworks, Inc., Natick, MA, USA) with an in-house program.

Absorbance measurements of each sample were processed as in [26]. Thus, bi-dimensional background was removed and each sample was represented by a peak counting table. Then, based on the chromatographic times at which the DNP-amino acids eluted, the time frame in both dimensions was limited: for the ^{1}D between 20 and 60 min, and for the ^{2}D between 0.9 and 10.9 s, and all peaks outside this bi-dimensional zone were discarded. Also, since the number of peaks detected in each sample varied, retention times were grouped (^{1}D: every 4 modulations, 32 bins; and ^{2}D: every 1 s, 10 bins) and peaks area summation was performed in the respective bins. Later, all non-zero values were replaced by one, thus the data were converted to its binary representation. This reduces the influence of the area differences between equivalent bins in different samples (i.e. peaks were present or absent), thus focusing on the position in the chromatographic space. Finally, the binary matrices of each sample, excluding the one with the amino acid standards, were transformed into vectors (each modulation on top of the other). Those vectors were grouped in a new matrix, which were analyzed with PCA. Since the data analyzed were binary, mean centering was not performed.

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3. Results and Discussion

3.1. ^{1}D Chiral separation optimization

The first step of the optimization process was focused in maximizing the enantioresolution for the individual DNP-amino acids standards in the ^{1}D by using the CSP-QN-EC column. This CSP has shown to be especially suitable for the separation of DNP-amino acid enantiomers. Based on previous results, the starting mobile phases were 50 mM ammonium formate buffer (pH 6.30) and MeOH under gradient elution. Within a pH range of 5 and 7, the quinuclidine moiety in the quinine has a positive charge (weak anion exchange behavior) whereas the DNP-amino acids are in anionic forms [22]. The buffer ionic strength and pH were kept constant. The optimization involved the gradient slope, the organic modifier (MeOH and ACN) and the column temperature.

The organic modifier had a different influence on the enantiomeric separation. Gradients with MeOH provided better enantioselectivities for most of the DNP-amino acids. On the other hand, lower enantioselectivity factors, although more symmetric and narrower peaks than those obtained with MeOH, were observed with ACN gradients. Thus, the optimization process consisted in testing incremental amounts of MeOH in the organic modifier to improve separation factors by starting at 100% ACN. Figure 2 shows the resulting selectivity (α) and the peak width (4σ) in the ^{1}D for a set of DNP-amino acids injected as pure standards. The final composition chosen as strong eluent in the ^{1}D mobile phase was a (50:50) MeOH:ACN mixture.

The use of an active pump in the interface allows to optimize the ^{1}D flow rate independently from the ^{2}D sampling time. Thus, the gradient time in the first dimension was determined by the optimal speed of enantioresolution of the amino acids plus glycine (Gly). A reasonable separation of most DNP-amino acids was obtained for a change in in composition of 2.5 %/min from 0 to 100% MeOH:ACN (50:50) in 40 min after 10 min under isocratic mode.

Enantioseparation factors as a function of column temperature have been evaluated. By increase of the temperature from 20 to 40 °C the retention times were significantly reduced by 66 % at the expense of a slight decrease in the enantioseparation under the same mobile phase compositions and gradient slope. However, the small loss of selectivity was more than compensated by higher efficiencies. Thus, 30 °C was selected for subsequent experiments.

Under these conditions practically all DNP-amino acid peaks were enantioseparated. Only Asn and Arg were very poorly resolved in the ^{1}D. Moreover, the number of derivatives obtained for Lys, Tyr and Orn (these amino acids that has more than one functional group to be DNP-labeled) was 5, 4 and 3 peaks, respectively. This is due to the presence of two reactive groups per molecule. For Orn only one was enantioresolved (one amine functionalized) and in the case of Lys two of them were enantioresolved and the bi-DNP-labeled were not enantioseparated. Tyr has a phenolic group and the amino group: Tyr1 and Tyr2 correspond to the DNP-derivatives onto the amino groups, and Tyr3 and Tyr4 are the bi-labeled derivatives that were enantioresolved.

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The chromatogram of the finally DNP-amino acids separations recorded in the ^{1}D detector is shown in Figure 3. The elution order, checked with some pure enantiomers, indicated that D-AA eluted after the L-enantiomer. It can be seen that the complete mixture of all compounds is unresolved: about thirty maxima can be counted. The compromise between peaks observed in this chromatogram suggests that this scenario cannot be improved simply by increasing the chiral column length. Overall, a sufficient ^{1}D chiral separation of the complete mixture was obtained in about 1 hour by the CSP-QN-EC column in their L-AAs and D-AAs, and all the eluent was on-line sampled and successively transferred to the second achiral column.

3.2. Optimization of the volume transferred

This simple active modulation scheme of splitting and dilution of the ^{1}D effluent can be implemented into the method optimization strategy. Splitting the flow after the first dimension column to perform online LC × LC on a constant fraction of the first dimension effluent allows the optimization of the two dimensions almost independently [27]. Moreover, two additional benefits by the use of a splitter pump should be mentioned: 1) it provides the flexibility to control the amount of sample transferred into the second dimension very precisely because this can be automated and adjusted as needed and, 2) since the flow splitter is implemented after the first dimension column, the reproducibility of the separation in the first dimension is not affected by any change in the split ratio. In this specific method, the active splitter pump was optimum at a flow-rate of 0.273 mL/min.

3.3. Optimization of the ^{2}D achiral separation

The second dimension (achiral) separation conditions should allow the fast separation of the components present on each fraction of the first dimension transferred. In this setup, that must be performed as fast as possible in order to keep the enantioresolution gained in the ^{1}D. Thus, the time elapsed in the second dimension analysis (cycle time) directly influences the volume being transferred and the undersampling of the first dimension. In fact, there is necessarily an optimum sample acquisition time which must be equal to the second dimension cycle time ($^{2}t_c$). The cycle time was optimized in 0.3 min and, for this reason, two 20 µL-loops were selected. The dilution of the sample was to a 40% with the incoming 2D eluent.

Conditions to speed up ^{2}D separation were a gradient elution, at high-flow rate to assure fast re-equilibration, and at temperature compatible with the stationary phase. In order to evaluate orthogonality respect to the ^{1}D retention mechanism, two column with different stationary phases were tested: a C18 and a phenyl-butyl column. Similarly, two acidic solutions were tested to evaluate the orthogonality of the separatons: perchloric acid and formic acid at pH 2. At this pH, the carboxylic groups of DNP-amino acids are protonated. No differences in retention times were noted with $HClO_4$ but some asymmetric peaks were observed (data not shown).

For the optimization of the organic modifier, pure ACN was selected due to the efficiency demonstrated in the ^{1}D. The column temperature and flow rate were optimized to reduce high pressure drops at the head of the ^{2}D

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column at the beginning of each gradient cycle. For 2D-achiral analysis the following conditions were applied after the optimization: flow rate 3 mL/min at 65 ^{0} C, mobile phases were A: 50 mM formic acid and B: ACN, using a gradient elution 0-70 % B in 0.25 min with a sampling time of 0.3 min.

The 2D-LC chromatograms (contour plots) obtained with the phenyl-butyl (A) and C18 (B) columns are shown in Figure 4. It is observable that in the zones limited between 45-55 min (^{1}D) and 6-10 sec (^{2}D) (enlarged picture) more peak resolution was achieved in the C18 column (Figure 4B) for the critical peak clusters. Moreover, 41 and 44 peaks could be distinguished by the software from the 2D images obtained in the phenyl-butyl and C18 column, respectively.

The peak assignment was made after injection of five groups of well separated amino acids. Figure 5 shows the bidimensional retention time graph of the identified DNP-amino acids. It can be seen that each amino acid elutes as a pair of peaks (L and D) except for Arg and Asn, which were not completely resolved in the ^{1}D and after the ^{2}D elution the poor enantioresolution was lost. It can also be noted that, as it was mentioned before, Orn, Tyr and Lys have more than one derivatized group and appears as mono- and bis-DNP labeled amino acids. For Orn, the bis-DNP labeled is not resolved (peak identified as Orn3), and in the case of Lys the five peaks become from the mono- and bis-DNP labeled amino acid enantioresolution whereas the fifth peak has been associated to an impurity in the amino acid standard. Tyr was derivatized in the amino group and on both, the amino and the hydroxyl groups, and the four peaks were separated. Most of the amino acids were well separated from each other, except for a few uncritical ones, which have been partially resolved, (Tyr1-Ile2-Ter2). In Figure 5 were also labeled the three extra peaks coming from the derivatization reagent which have been confirmed with the injection of the reaction blanks.

The fact that separations are performed by two distinct complementary mechanisms in ^{1}D and ^{2}D provides enhanced assay specificity because each of the two dimensions can resolve some impurities. This becomes clearly evident in the chromatograms, also considering the fact that a specific precolumn derivatization is necessary to increase sensitivity and also enough RPLC retention of AAs. Of importance for the method validity is the evaluation of retention time stability over several sample injections. This has been tested by replicate injections of amino acid standard mixtures and the run-to run repeatability in this LCxLC is below 5 % RSD for n=10 for all DNP-amino acids.

3.4. Application for qualitative analysis of honey samples

Honey samples were collected from thirteen different regions in Mendoza Province (Argentina) that are detailed in Table 1. Figure 6 shows the 3D plots corresponding to the thirteen honey samples 2DLC plots and Table 1 summarized the amino acid identified in each sample. Also, the number of peaks observed for each sample is reported in Table 1. The counting procedure is based on the report given by the developed software and the peaks identification was done by visual inspection of retention times of each individual bidimensional chromatogram as compared to the corresponding to the DNP-amino acids standards.

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3.5. Principal Component Analysis

Peak counting was performed on the chromatographic data for each honey sample. After the binning of retention times, the data were converted to their binary representation and PCA was performed. Results can be seen in Figure 7 The scores of each honey sample in the PC1 x PC2 plane are shown in Figure 7-A). In the PC1 direction, almost 50 % of the variance is explained. Although all scores are positive, the samples are distributed. In the PC2 direction, samples 11-13 are grouped in the zone of positive scores. The remaining samples have negative or close to zero values.

In order to evaluate which DNP-amino acids were the most significant to discriminate different honey samples in the scores plot, the coefficients of each PC can be analyzed. In figure 7-B), it can be observed that for PC1, all the ovals are red, which means that the most significant coefficients were positive. This is expected given that mean centering was not performed before to PCA and that all scores were also positive in PC1. The location of the selected ovals can be related with the peaks that most influenced the discrimination among samples meanwhile the relative size of each oval is an indicator of the magnitude of the respective influence. Most of the significant coefficients are seen in the lower-left quadrant, which includes Pro1/Pro2, Ala1/Ala2, Gly, Leu1, Lys3, Val1, Asn, Arg, and Lys1. In the upper-right quadrant, Thr1, Ile1, and Lys4 were also selected. On the side of PC2, Figure 7-C) shows that most of the significant positive coefficients were not associated to DNP-amino acids, but they were related to other components in the honey samples (considering that other honey components may also have reacted with the DNPF). On the other hand, significant negative coefficients were selected for DNP-amino acids. With the exception of Nor1, the rest of them (Lys1, Asn, Ile1 and Ala2) were also significantly positive in the PC1, which implies that some direct relationships, in terms of presence/absence, cannot be easily established. This can be attributed to the high complexity of the analyzed data, the effect of peak shifts that the binning strategy may have not be solved, the fact that the DNP-amino acid standards sample was not included in the PCA and that it was only utilized to obtain a binary two-dimensional reference chromatogram.

Although the separation and identification of enantiomeric amino acids is already established in the chromatograms, it is not the same with respect to the complexity of the sample and the selectivity of the method. The contributions of the application of the developed LCxLC method are the separation and tentative identification in a single chromatographic run of the DNP-amino acids profiles in honey samples by PCA. To the best of our knowledge, this is the first time that chiral x achiral separation of DNP-amino acids is done using a 2D-LC method (LCxLC), and its application to enantiomeric amino acid analysis in honey samples.

4. Conclusions

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The development of a new two-dimensional comprehensive chiral × achiral RPLC method to determine the presence of amino acids in honey samples was presented. This method included the use of a chiral column based on quinine stationary phase for the enantiomeric separation in the first dimension and a C18 porous column to carry out the RPLC separations in the second dimension. This combination provided enough orthogonality to the multidimensional method to resolve twenty DNP-amino acids, including most enantiomers. The use of an active splitter pump in the modulator allowed us to: i. optimize the ^{1}D flow rate independently from the ^{2}D cycle time and ii. make a judicious control of the sample volume transferred into the ^{2}D.

To the best of our knowledge, *this is the first time that an on-line two-dimensional comprehensive LC is applied to the enantiomeric separation of amino acids in such a complex natural sample by using the fully comprehensive configuration.* The method developed may be of usefulness for bioanalytical investigations of minor quantities of D-amino acids in presence of large amounts of L-amino acids in biological samples where traces of interferences could be also more critical than in honey samples.

Acknowledgements

This work was supported by the following government institutions from Argentina: ANPCYT (PICT2016#2662), UNLP (11X/839 and 11X/836) and CONICET. The authors appreciate the support from Agilent Technologies GmbH for providing HPLC modules and for their permanent technical support.

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Figure Legends

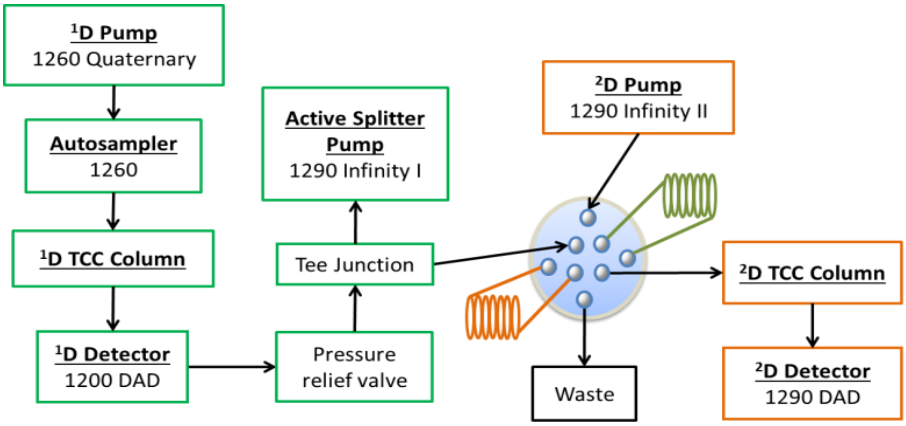

Figure 1. Diagram of the module configuration and flow direction of the full comprehensive 2DLC system. The green boxes represent the first dimension and in orange the second dimension system.

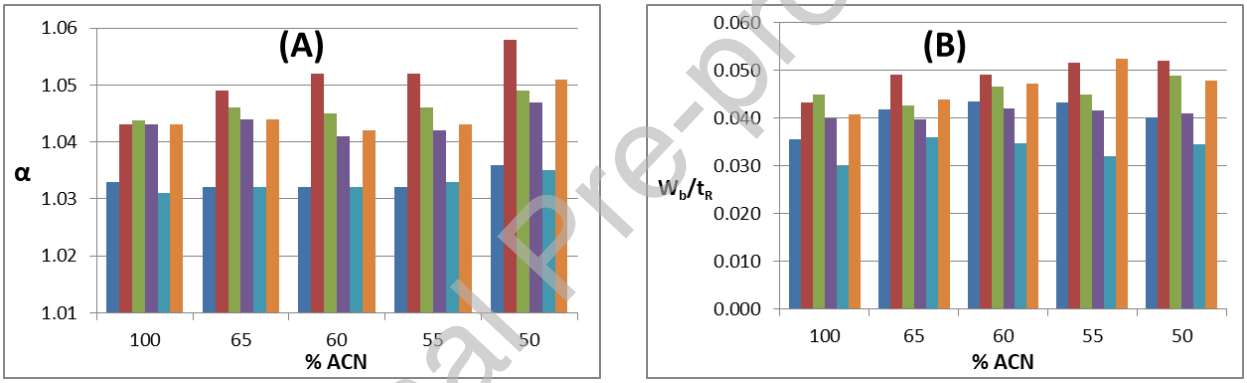

Figure 2. Plots of enantioselectivity (A) and peak width (W_b/t_R) (B) of six representative DNP-amino acids at different composition of organic modifier from 100% ACN to 50:50 % ACN:MeOH. The solute identifications are (from left to right): DNP-His (blue); DNP-Val (red); DNP-Leu (green); DNP-Pro (purple); DNP-AB (light blue) and DNP-Ser (Orange). Chromatographic conditions were: 0.3 mL/min, 30 ^{0} C and a gradient elution from 0-100 %B in 40min. Solvent A was 50mM ammonium formate pH 6.30. The %RSD values for both parameters plotted were lower than 0.5% (n=3).

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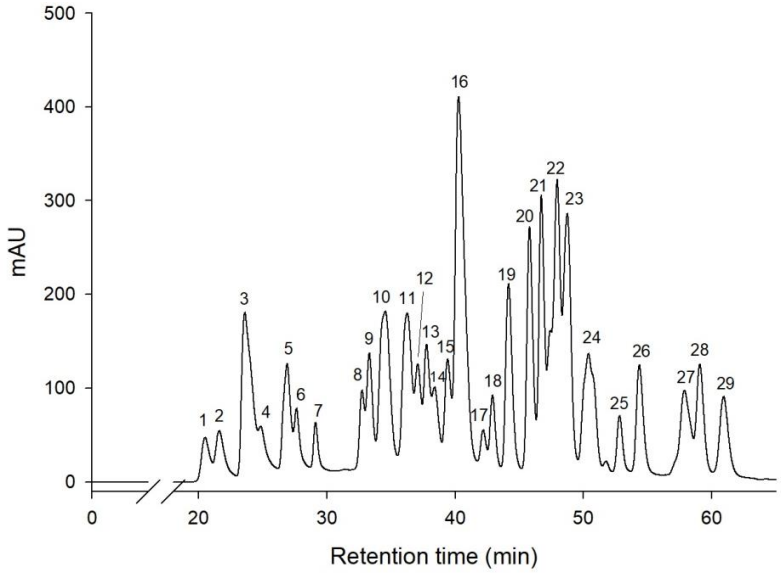

Figure 3. Chromatogram of the DNP-amino acids standard solution separated in the chiral column and recorded at the [1]D DAD. Peaks identification: 1= Orn1; 2= Orn2; 3= Orn3; 4= unknown from Derivatization Reaction (FDR); 5= Arg; 6= Lys1; 7= Lys2; 8= Lys3; 9= Asn; 10= Val1+Leu1; 11= Val2+Leu2; 12= His1; 13= Pro1+His2; 14= FDR; 15= Pro2; 16= Gly; 17= Ala1; 18= Ala2; 19= AB1; 20= Thr1+AB2; 21= Ter1+Thr2; 22= Ter2+Ile1+Met1; 23= Ser1+Met2+Tyr1+Ile2; 24= Tyr2+Nor1+Phe1+Ser2; 25= Nor2; 26= Trp1; 27= Tyr3+Trp2+FDR; 28= Lys4+Tyr4 and 29= Lys 5. Chromatographic conditions: Gradient: 0 (10min) – 100% B in 50 min. A: 50 mM ammonium formate pH 6.30 and B: 50:50 ACN:MeOH; Temperature: 30 °C; Flow rate: 0.3mL/min. Injection volume: 8µL.

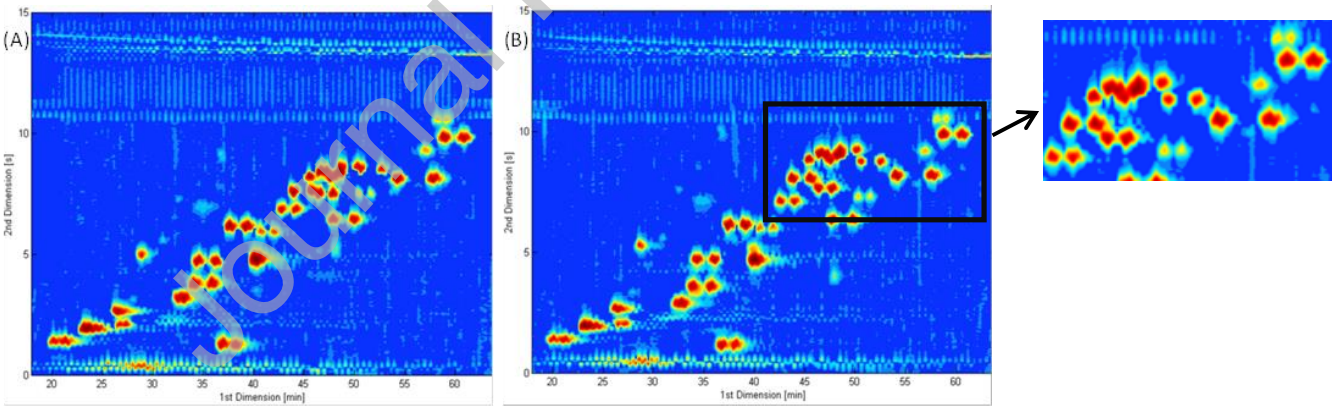

Figure 4. Contour plots of the chiral x achiral separation of the DNP-amino acids in a phenyl (A) and a C18(B) column under the optimum chromatographic conditions for each dimension: [1]D: Gradient: 0 (10min) – 100 % B in 50 min. A: 50 mM ammonium formate pH 6.30 and B: 50:50 ACN:MeOH; Temperature: 30 °C ; Flow rate: 0.3mL/min. [2]D: flow rate 3 mL/min at 65 °C, mobile phases were A: 50 mM Formic acid and B: ACN, using a gradient elution 0-70% B in 0.25min. Cycle time: 0.3 min.

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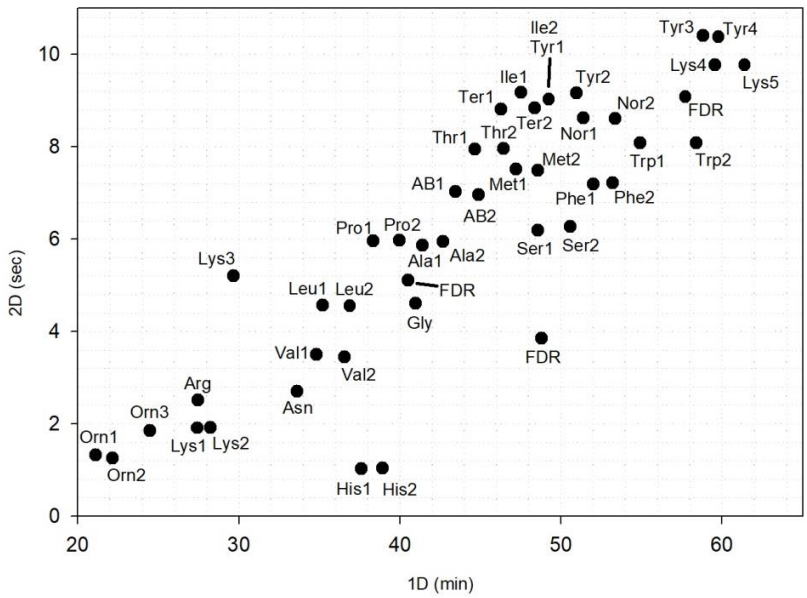

Figure 5. Map of retention times in both dimensions of the DNP-amino acids corresponding to the standard solutions. FDR: peaks from derivatization reagent. Chromatographic conditions as in Figure 4.

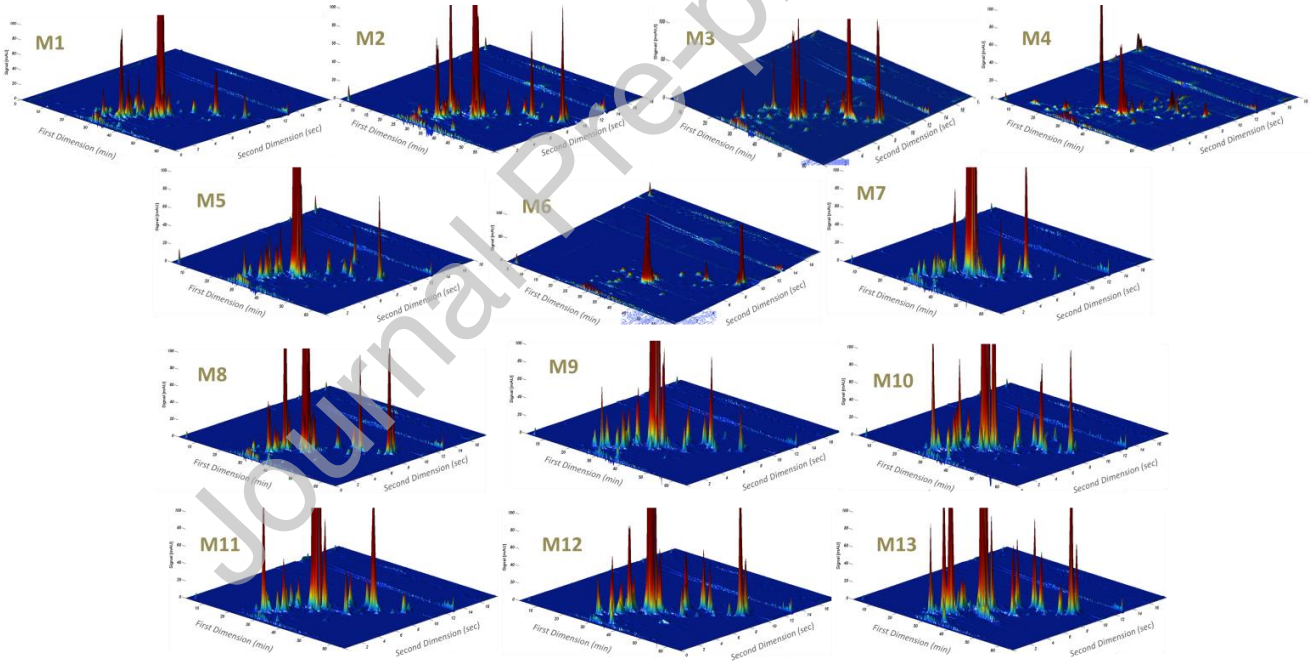

Figure 6. 3D plots of thirteen honey samples, labeled M1 to M13, from different regions of Mendoza province (Argentina), as they were detailed in Table 1.

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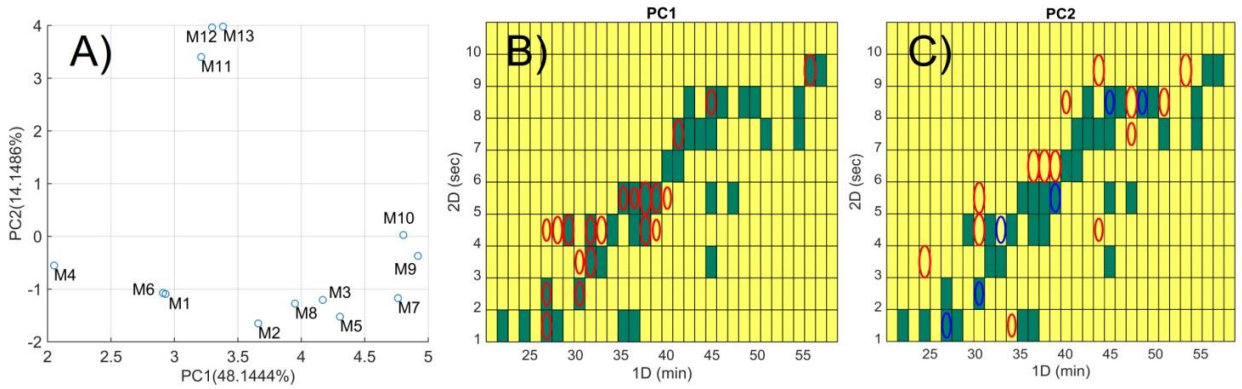

Figure 7. Principal Component Analysis on honey samples. A) PC1 x PC2 scores plot (explained variances in parentheses); B) Visualization of the 20 most significant coefficients of PC1, projected on the binary representation of Figure 5 (yellow and green for DNP-amino acids absence and presence, respectively); C) the same as in B), for PC2. In B) and C), the area of each oval is proportional to the respective coefficient scale factor (see section 3.5.), while the color is red or blue for positive or negative coefficients, respectively.

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Table 1. D- and L-amino acids identified in thirteen honey samples (M1-M13) from different regions of Mendoza (Argentina).

	Place of Origin (Mendoza Province)	N° of Peaks*	Orn3	Lys1	Lys3	Arg	Asn	His1	Leu1	Leu2	Val1	Val2	Pro1	Pro2	Gly	Ala1	Ala2	GABA1	GABA2	Thr1	Tert1	Tert2	Tyr2	Ile1	Ile2	Ser1	Nor1	Nor2	Phe1	Trp1	Lys 4	Lys5
1	Potrerillos	45		x	x		x	x		x		x	x				x	x							x			x	x		x	x
2	Potrerillos	49		x	x		x		x	x	x		x				x		x			x			x		x	x				x
3	Rivadavia	51		x	x						x	x	x			x	x		x			x		x	x		x				x	x
4	Potrerillos	44		x	x						x	x	x				x	x									x					x
5	Chacras de Coria	47		x	x		x		x	x	x		x		x	x	x	x		x		x		x		x	x			x	x	
6	Mayor Drummond, Luan de Cuyo	29		x	x		x	x			x		x				x			x		x					x		x	x	x	
7	El cepillo, Joan Carlos	53	x	x	x	x	x				x				x							x					x		x	x	x	
8	Malargüe	45		x	x			x			x		x	x		x	x	x						x			x				x	
9	Lulunta, Maipú	47	x	x	x	x		x	x				x	x		x					x			x		x	x	x		x	x	
0	La Barrancas, Maipú	50	x	x	x	x		x	x				x	x	x	x				x				x			x			x	x	
1	Colonia Segovia-Guaymallén	45	x	x	x	x		x					x	x	x	x							x	x		x				x		
2	Tareditas, San Carlos, La Villegas	46		x	x			x					x	x		x				x			x						x		x	
3	Perdriel	63		x	x	x		x				x	x	x	x	x				x				x		x		x	x		x	

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* The number of peaks found in each chromatogram is indicated.

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