SHIMADZU
Excellence in Science

Technical Report

Continuous *vs.* Segmented Second Dimension System Gradients for Comprehensive Two-dimensional Liquid Chromatography of Sugarcane (*Saccharum spp.*)

LC×LC for polyphenol analysis in sugarcane

Gabriel Mazzi Leme[1], Francesco Cacciola[1], Paola Donato[1], Alberto Jose Cavalheiro[1], Paola Dugo[1,2], Luigi Mondello[1,2]

Abstract:

Polyphenols in sugarcane (*Saccharum spp.*) leaf extracts were analyzed by comprehensive two-dimensional liquid chromatography in combination with photodiode array and mass spectrometry (LC×LC-PDA-MS) detection; a micro cyano and a partially porous octadecylsilane column were employed in the first and the second dimension, respectively. Even using RP mode in both dimensions, a satisfactory degree of orthogonality was achieved by employment of different approaches of gradient elution mode in the second dimension. By means of the investigated set-up, a total of 38 polyphenolic compounds were detected with the complementary data of PDA, MS and an in-house database.

Keywords: polyphenols, LC×LC, mass spectrometry, cyano column, sugarcane

1. Introduction

Sugarcane belongs to the Poaceae family and to *Saccharum* genus and it is represented by the species *S. officinarum* L., *S. spontaneum* L., *S. robustum* J., *S. sinensis* R., *S. barberi* J. Interspecific crosses allowed the development of modern varieties with improved agronomic traits such as disease resistance and high sugar production.

A large amount of compounds have already been identified and described for wild species and commercial varieties of sugarcane, mainly phenolic compounds as flavonoids *O* and *C*-glycosides and anthocyanins. Despite a few reports dealing with the metabolic composition of sugarcane, the large amount of bioactive compounds in sugarcane has not yet been properly characterized.

The use of hyphenated techniques *e.g.* comprehensive two-dimensional chromatography (LC×LC) could be a viable tool for polyphenol characterization in complex plant extracts. The main advantage of these techniques compared to the traditional methods is the increased peak capacity due to different retention mechanisms in each dimension. On-line coupling of the LC×LC system to photodiode array (PDA) and mass spectrometry (MS) detection allowed the attainment of a thorough metabolic fingerprint of such a promising crop (Fig. 1).

2. Experimental

2-1. Samples and Sample Preparation

Sugarcane leaves (cultivar RB3280), were collected from a commercial plantation in Araraquara city (São Paulo State, Brazil), dried in an oven (40 °C) during 48 hours and milled in a knife mill. The extracts were prepared using 200 mg of dried material with three consecutive 2.0 mL aliquots of a water/ethanol/isopropanol (30:45:25, *v/v/v*) mixture in an ultrasonic bath (Unique, model USC-2800, 40 kHz, 120 W, 7.8 L) during 15 minutes under ambient temperature. The combined extracts were dried and then dissolved in a water/methanol (1:1, *v/v*) mixture to get a 50 mg/mL solution that was subjected to filtration through a 0.45 µm Acrodisc nylon membrane filter (Pall Life Sciences, Ann Arbor, MI, USA) and stored in a 1.5 mL vial.

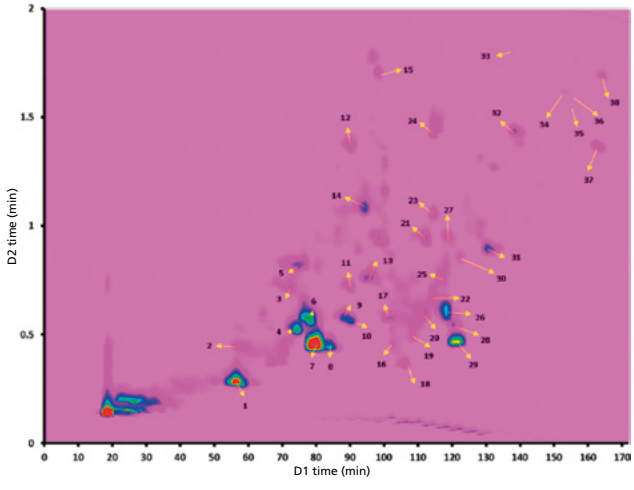

Fig. 1 RP-LC×RP-LC Plot of a sugarcane extract.

1 University of Messina, Italy
2 Chromaleont S.r.l.

2-2. Reagents and Materials

For the extraction procedure, ethanol and isopropanol HPLC grade were provided by J. T. Baker Solusorb (Xalostoc, Mexico). The water used was purified in a Millipore Milli-Q system (Millipore Corp, New Bedford, MA, USA). For LC×LC-PDA-MS analyses, ethanol, LC grade was purchased from Sigma-Aldrich (Gillingham, United Kingdom), water and methanol LC-MS grade and acetic acid (99%) were purchased from Riedel-de Haën (Seelze, Germany).

Chromatographic separations were carried out using different columns provided by Supelco (Bellefonte, PA, USA): Ascentis Cyano and Ascentis RP-Amide (250 mmL. × 1 mmI.D., 5 µm d.p.), and Ascentis Express C18 (30 mmL. × 4.6 mmI.D., 2.7 µm d.p.).

Standards of gallic acid, chlorogenic acid, catechin, caffeic acid, vanillin, coumarin, naringenin, cinnamic acid were provided by Sigma-Aldrich (St. Louis, MO, USA) and rutin, quercetin, luteolin, apigenin and kaempferol were provided by Extrasynthèse (Genay, France). The standard mixture employed for the selectivity evaluation was prepared from the individual solutions (1 mg/mL) and the final concentration of each standard was approximately 0.08 mg/mL.

2-3. LC×LC Instrumentation and Software

- Shimadzu CBM-20A controller
- two Shimadzu LC-20AD dual-plunger parallel-flow pumps
- Shimadzu LC-20AB dual-plunger parallel-flow pumps
- Shimadzu DGU-20As degassing unit
- Shimadzu CTO-20A column oven
- Shimadzu SIL-20AC autosampler
- Shimadzu SPD-M20A photo diode array detector (2.5 µL detector flow cell)
- Shimadzu LCMS-2020 mass spectrometer

For connecting the two dimensions: 2-position 10-port switching valve (Supelco, Bellefonte, PA, USA) placed inside the column oven and equipped with two identical 20 µL sample loops.

2-4. Software

- Shimadzu LabSolutions (Version 5.41 SP1)

2-5. 2D Software

- ChromSquare (Version 2.0) from Chromaleont, Messina, Italy

3. LC×LC-MS Conditions

D1 separations: Ascentis Cyano column

Flow rate	: 20 µL/min
Mobile phases	: (A) water/acetic acid (99.9/0.1 *v/v*) and (B) ethanol/acetic acid (99.9/0.1 *v/v*)
Gradient elution	: 0 min, 10% B; 120 min, 40% B; 130 min, 100% B; 172 min, 100% B.
Injection volume	: 5 µL

D2 separations: Ascentis Express C18 column

Mobile phases	: (A) water/acetic acid (99.9/0.1 *v/v*) and (B) methanol/acetic acid (99.9/0.1 *v/v*)
Flow rate	: 3.0 mL/min
Modulation time of the switching valve	: 2 min

Gradient elution:

FIF, full in fraction: 0.01 min, 5% B; 0.10 min, 5% B; 1.50 min, 50% B; 1.51 min, 100% B; 1.80 min, 100% B; 1.81 min, 5% B; 2.01 min, 5% B

SIF, segmented in fraction: (0–100 min): 0.01 min, 15%B; 1.50 min, 25%B; 1.80 min, 25%B; 1.81 min, 15%B; 2.01 min, 15% B; (100–172 min) 0.01 min, 25% B; 1.50 min, 35% B; 1.80 min, 35% B; 1.81 min, 25% B; 2.01 min, 25% B.

CS, continuously shifting: 0.01 min, 5% B; 172 min, 30 % B.

MS conditions

MS acquisition performed using the ESI interface operating in both positive and negative ionization modes:

mass spectral range: 200–800 *m/z*; event time: 1 sec; scan speed: 625 u/s; nebulizing gas (N_2) flow: 1.5 L/min; drying gas (N_2) flow: 15 L/min; Heat block temperature: 300°C; desolvation line (DL) temperature: 300°C; DL voltage: 0V; probe voltage: –4.5 kV; Qarray DC voltage: 0 V; Qarray RF voltage: 100 V; detection gain: 1.0 kV. The flow eluting from the second column was split before the MS instrument (approximately 0.4 mL/min to the MS).

4. Results and Discussion

Comprehensive two-dimensional liquid chromatography was employed to get a metabolic fingerprint of sugarcane leaves. The use of reversed phase in both dimensions avoided mobile phase incompatibility issues but on the other hand, it offered limited separations due to the lack of orthogonality, which was overcome by the use of different gradient elution approaches for the second dimension separation.

5. Selectivity Correlation

In order to get some degree of orthogonality, selectivity correlation plots were investigated employing different combinations of columns (cyano, amide and C18 stationary phases) and mobile phases (MeOH and EtOH as B solvent) with a solution of reference materials reported in the Reagents and Materials section. The unusual employment of ethanol as mobile phase is an option when more environmentally friendly solvents are desired, especially in cases with high consumption of solvents. From the results attained, the smallest value of correlation between the columns evaluated was obtained when ethanol was used as B solvent in both dimensions (Table 1). Despite this, due to its higher viscosity which resulted in higher backpressure values, this solvent could not be used at high flow rates for fast D2 separations, since conventional LC instrumentation was employed (P_{max} = 440 bar). As a consequence, the combination of cyano and C18 columns, using EtOH and MeOH as B solvent, respectively for D1 and D2 was selected for the subsequent LC×LC separations.

Table 1 Regression coefficients (R^2) for the correlation selectivity plots for different combinations of stationary mobile phase employed

	Ascentis Express C18 (D2)			
	MeOH (D1/D2)	EtOH (D1/D2)	MeOH (D1) × EtOH (D2)	EtOH (D1) × MeOH (D2)
Ascentis Cyano (D1)	0.907	0.585	0.705	0.761
Ascentis RP Amide (D1)	0.848	0.847	0.732	0.776

6. LC×LC Separations for Polyphenolic Analysis in Sugarcane Leaf Extracts

Fig. 2 shows the RP-LC×RP-LC plots of the sugarcane leaves extract by employing three different set-ups, using the optimized conditions for each dimension, on the basis of the selectivity evaluation.

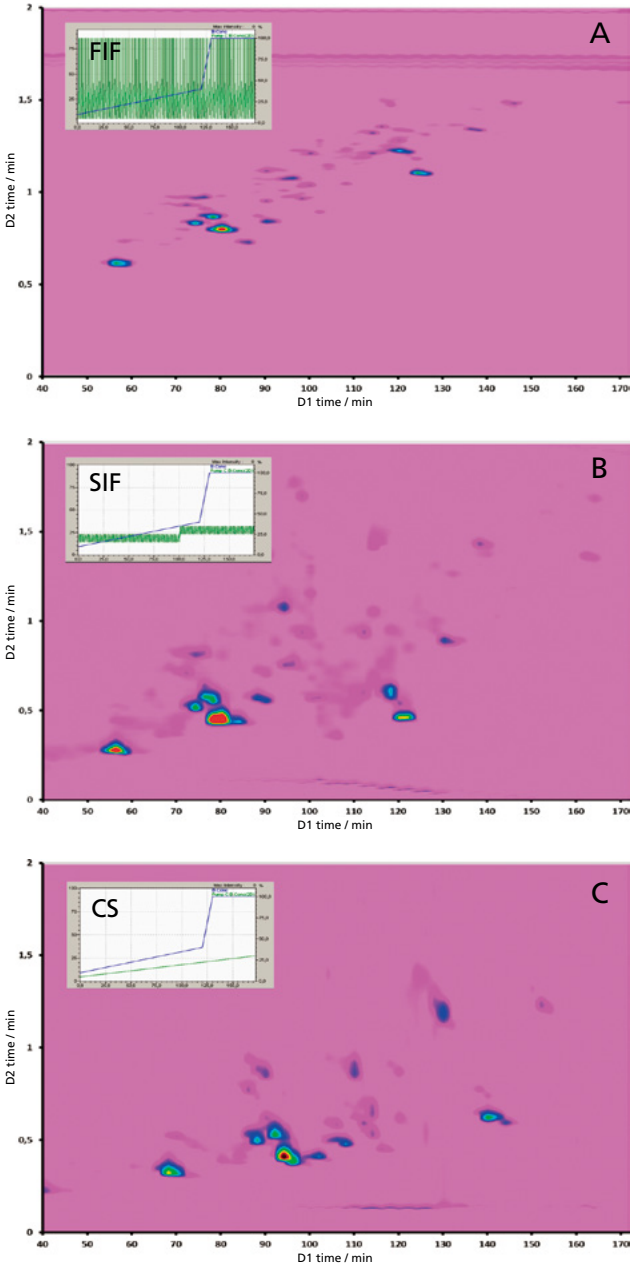

Fig. 2 RP-LC×RP-LC contour plot of the sugarcane extract investigated in this work, by means of set-up 1 (A), set-up 2 (B) and set-up 3 (C).

The first approach used, known as full in fraction (FIF), is the most commonly adopted for LC×LC separations (set-up 1). Such a method involves an equally generic and steep mobile phase range in each repeated D2 run time. As it can be seen in Fig. 2A, despite the partial correlations of the columns employed, a good separation of most of the compounds in this complex sample was attained.

In order to improve the peak distribution, different gradient elution strategies were investigated to enhance the orthogonality degree, by means of specific elution gradient approaches to be used in the D2. The first one, segmented in fraction (SIF) employed different gradient ranges in different segments of the D1 separation (Set-up 2). For the sample analyzed in this work, two different repetitive gradients were tested during the entire LC×LC retention window.

The resulting RP-LC×RP-LC plot of the sugarcane leaves extract (set-up 2) is illustrated in Fig. 2B. From a visual inspection, it can be appreciated how the overall D2 separation space was more considerably covered with respect to the one attained by set-up 1.

Aiming to improve the overall peak distribution a second strategy, known as Continuously Shifting (CS), was investigated. In such an approach, only one single gradient spanning the whole D2 separation time was adopted simultaneously to the D1 gradient. Under such conditions, every fraction in D2 was analyzed in a very small gradient step (D approximately equal to 0.3 %B). As can be seen in Fig. 2C, a higher separation space was occupied in comparison to set-up 1, but despite of it, less compounds can be detected due to inefficient peak focusing.

All the analyses were carried out in triplicate to evaluate the repeatability of the system. The relative standard deviations were smaller than 5% for the retention time of selected peaks.

7. Evaluation of the LC×LC Separations

An evaluation of the performance, in terms of peak capacity (n_C), of the different set-ups tested was carried out. For such calculations the n_C values of the single dimensions were calculated using the well-known method defined by Neue. A total of nine chromatographic peaks were selected for the calculations, arising from representative components eluting over the LC×LC retention scale.

The theoretical peak capacity values, being multiplicative of the individual values obtained for the two dimensions (${}^1n_C \times {}^2n_C$) for the three set-ups tested were 1380, 720 and 780 respectively.

To attain more realistic peak capacity values, another approach was considered known as "practical" peak capacity values corrected for both undersampling (number of fractions effectively transferred from the D1 to the D2) and orthogonality (separation space effectively covered by the sample components).

Set-up 2, attained by the use of the segmented gradient, turned out to be the most efficient one since it suffered less from the correlation of the two dimensions tested. Such an approach clearly shows how, despite the use of partially correlated systems, a considerable increase in efficiency can be attained by the use of careful gradient strategies in LC×LC (Table 2).

Table 2 Relative performances, in terms of peak capacity, n_C, of the three instrumental set-ups developed

	SET-UP #1	SET-UP #2	SET-UP #3
${}^1n_C$	30	30	30
${}^2n_C$	46	24	26
Theoretical D2 n_C	1380	720	780
Practical D2 n_C	129	308	256

8. Conclusions

A novel comprehensive two-dimensional liquid chromatography system, based on the use of a micro cyano column and a partially porous (C_{18}) column in first and second dimension, respectively, in combination with photodiode array and mass spectrometry detection, is presented.

Among the second dimension strategies tested, the employment of a segmented gradient (set-up 2) turned out to be the most effective one, yielding the highest peak capacity allowing to detect 38 polyphenolic compounds and among them 24 were positively identified in the sugarcane leaves extract analyzed by means of the complementary data of PDA, MS and an in-house database.

The proposed method offers new possibilities in metabolomic studies that can be extremely relevant in the understanding of plant metabolism in different growing conditions.

First Edition: December, 2015

Shimadzu Corporation
www.shimadzu.com/an/