



## Original Article

# Coupled monolithic columns as an alternative for the use of viscous ethanol–water mobile phases on chromatographic fingerprinting complex samples

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## ABSTRACT

Concepts of sustainability have received attention from people involved in investigation of nature-derived matrices. The effects of concomitant pollutant activities are cumulative and harmful to the environment from which these matrices are obtained. High performance liquid chromatography analyses generate millions of liters of chemical waste worldwide every year. Reduction of organic solvent consumption during the analyses and replacement of harmful solvents with greener options are the main approaches to mitigate this problem. This work explored the strategy of employing monolithic columns when the problematic acetonitrile is intended to be replaced with the greener but more viscous ethanol in fingerprinting a leaf extract of *Lippia sidoides* Cham., Verbenaceae, by high performance liquid chromatography. Two monolithic columns were coupled in series to test a more critical backpressure condition while doubling the number of theoretical plates, which can be useful to separate the hundreds of compounds present in plant extracts. All work was conducted by employing design of experiments. A mathematical model indicated an optimum point in which ethanol was the only organic solvent of the mobile phase. However, the use of a proper metric, which considered environmental parameters together with separation parameters, evidenced that an experimental condition of the original central composite design should be preferred over the former even if containing 20% acetonitrile in the organic modifier mixture. Flow rates of up to 3 ml/min were accommodated with two coupled monolithic columns without exceeding 250 bar. These findings reinforced that no state-of-the-art instruments are needed to shift from traditional harmful solvents to greener ones, but only require a shift in researchers' approach toward sustainability.

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## Introduction

Concepts of sustainability have obtained increasing attention from people involved with analytical techniques, such as analytical, natural products, and food chemists (Castro et al., 2018; Funari et al., 2014a; Tobiszewski and Namieśnik, 2017). The effects of concomitant and repetitive pollutant activities tend to be cumulative and harmful to the environment. As such, it does not matter, if these concomitant activities are of the same nature or not, or if they are performed in analytical scale or larger. Moreover, environmental and occupational safety legislation have increasingly imposed more restrictive regulations, thus hindering the continuation of

unsustainable practices even at the analytical scale (Tobiszewski et al., 2010).

In this context, green analytical chemistry (GAC) has emerged as a branch of green chemistry (Gałuszka et al., 2013; Tobiszewski and Namieśnik, 2017). The first works to address GAC procedures appeared in 1995 (Guardia and Garrigues, 2011). Later, entire journal issues, books, and other initiatives have been addressed to GAC. The creation of a commission by the US Environmental Protection Agency (EPA) for screening and approving analytical procedures for different purposes was an important step in this direction (Tobiszewski et al., 2010).

Among the analytical techniques, high performance liquid chromatography (HPLC) deserves special attention in this discussion due to the fact that HPLC analyses generate 26,000,000–52,000,000 liters of chemical waste worldwide every year (Pereira et al., 2010; Welch et al., 2010; Shen et al., 2015; Sutton et al., 2018).

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Acetonitrile (MeCN) is the major organic modifier of choice due to its favorable chromatographic characteristics, such as low viscosity compared to methanol (MeOH) and ethanol (EtOH) (Funari et al., 2015). However, it is problematic from the sustainable point of view and any effluent containing MeCN must be treated as chemical waste (Welch et al., 2010; Prat et al., 2016).

Two main approaches have been adopted to avoid problems related to harmful solvents in HPLC and related techniques, which are: (i) reduce consumption of organic solvent during the analysis and (ii) eliminate or replace harmful solvents with greener options, such as EtOH and acetone (Welch et al., 2010; Funari et al., 2015). Strategy “i” can be achieved both by enhancing the elutropic strength of water when heating the mobile phase (this technique is called high temperature liquid chromatography – HTLC) and/or by scaling down the process of separation (Sandra et al., 2010; Welch et al., 2010; Plotka et al., 2013). There is a new generation of sub-2- $\mu\text{m}$  fully porous and sub-3- $\mu\text{m}$  core-shell particles packed columns with the height of theoretical plate (HETP) as low as 3.4–3.5  $\mu\text{m}$ , which allows reduced flow rate and analysis time without loss of separation performance (Gritti and Guiochon, 2012; Plotka et al., 2013). The main drawback of such approaches is the need for technological updating and thus financial investments, which are not always affordable for small laboratories (Shaaban and Górecki, 2012; Plotka et al., 2013). When a separation is performed close to room temperature, an ultra-high pressure liquid chromatograph (UHPLC) is needed to flow up the mobile phase through a small packed column. On the other hand, HTLC requires at least a mobile phase pre-heater and a thermostated column compartment (Shaaban and Górecki, 2012; Plotka et al., 2013; Funari et al., 2014b). The greatest advantage in approach “ii” outlined above is that it could lead to the end of HPLC analysts’ daily contact with harmful solvents. A more innovative approach has just been proposed by Sutton et al., who proved the possibility of employing natural deep eutectic solvents (NADES) as the major mobile phase components in HPLC analyses (Sutton et al., 2018). This finding opens many possibilities of new mobile phase for HPLC other than those based on traditional solvents such as MeCN and MeOH. While completely eliminating organic solvent in the mobile phase by employing pure water is a promising approach, the temperature required to elute non-polar components with pure water is currently prohibitive. Furthermore, selectivity appears to be a main issue and new developments in thermostable stationary phase and instrument technologies will be required to achieve this aim (Causon et al., 2012).

EtOH appears as the most promising green organic solvent for HPLC or UHPLC to replace harmful solvent, since it presents good physical properties for LC (it is miscible with water, less volatile, and less toxic than MeOH and MeCN and has a UV cut-off of 210 nm) (Sandra et al., 2010; Welch et al., 2010; Funari et al., 2014b). According to Welch et al. (2010), the use of the biodegradable EtOH together with all other environmental compatible chemicals during the HPLC analyses opens the opportunity of performing analyses outside the laboratories boundaries (Welch et al., 2010). The development of *in situ* analyses is a principle of GAC (Gałuszka et al., 2013). The widespread availability of EtOH, which is also produced from renewable feedstock, suggests that this solvent should be preferred in analyses carried out in environmentally sensitive areas (Welch et al., 2010). The same applies for developing places where chemical waste disposal is not yet well established or its price is prohibitively expensive (Welch et al., 2010).

The relatively high viscosity of EtOH, compared to those of MeOH, MeCN and their mixtures with water, has been pointed out as the main technical drawback of EtOH for LC application (Welch et al., 2010; Plotka et al., 2013). However, at least four strategies can be used to keep backpressures below the limit for a given LC system when the greener EtOH is used to replace MeCN or MeOH: (a)

reducing the mobile phase flow rate during the analysis (Funari et al., 2014a); (b) employing a monolithic column, which has a continuous homogenous phase bed instead of a packed bed with individual particles (Destandau and Lesellier, 2008). It generates low flow resistance to the mobile phase due to its higher porosity (Destandau and Lesellier, 2008); (c) pre-heating the mobile (and stationary) phases to decrease its viscosity, which is the case of HTLC (Funari et al., 2014b); and (d) adding high proportion of liquefied gases (such as  $\text{CO}_2$  or  $\text{CHF}_3$ ) in a mobile phase to reduce its viscosity (this technique is called *enhanced fluidity liquid chromatography*) (Pereira et al., 2010). Strategies a and b are the easiest and cheapest options because they do not require any technological improvement of a conventional instrument (Funari et al., 2014a). On the other hand, strategies c and d require some instrument adaptation to obtain HTLC conditions and to keep the entire mobile phase liquefied, respectively (Pereira et al., 2010; Funari et al., 2014b). Strategies a and c were successfully employed by our group to replace MeOH and MeCN with EtOH on the fingerprinting studies of *Casearia sylvestris* Sw. and *Bauhinia forficata* Link; and *Bidens pilosa* L. leaf extracts, respectively (Funari et al., 2014a,b), whereas strategy b was explored by Destandau and Lesellier (2008) to separate sunscreen and triazines (Destandau and Lesellier, 2008). Later, Yehia and Mohamed (2016) applied this strategy to separate a mixture composed of phenylephrine, paracetamol, and guaifenesin (Yehia and Mohamed, 2016).

In our on-going effort to develop green strategies that are compatible with the analytical platforms available for the largest number of natural products chemists, the strategy of employing monolithic columns, when the problematic MeCN is intended to be replaced with the greener but more viscous EtOH to separate a complex extract (aerial parts of *Lippia sidoides* Cham., Verbenaceae), was explored in this work. Two monolithic columns were coupled in series to test a more critical backpressure condition while doubling the number of theoretical plates, which can be useful to separate hundreds of compounds commonly present in plant extracts. A HPLC system capable of accommodating a maximum backpressure of 300 bar was used. A metric which encloses parameters related to separation and those related to sustainability was employed to properly compare the statistically selected separation conditions tested in this work.

## Material and methods

### Chemicals

The EtOH and MeCN (J.T. Baker, USA) were HPLC grade. The acetic acid (Synth, Brazil) was AR (ACS) grade.

### Plant material

Aerial parts of *Lippia sidoides* Cham., Verbenaceae, were collected at the Federal University of Ceará (UFC, Pici campus) in July 2011. Voucher specimen was deposited in the Herbarium “Prisco Bezerra” of the UFC under accession number 49.108.

### Extraction and concentration

*Lippia sidoides* aerial parts were dried at 30 °C in an oven with air circulation and ground in a knife mill. A mass of 15.1 g of the dried ground material was extracted by maceration with three aliquots of 50 ml of EtOH at 30 °C, with constant stirring. The fluid solutions were put together and concentrated at reduced pressure at 35 °C in a rotatory evaporator.

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