



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 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- μm fully porous and sub-3- μm core-shell particles packed columns with the height of theoretical plate (HETP) as low as 3.4–3.5 μm , 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 CO_2 or 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.

Table 1
Three factor central composite design (runs 1–19), optimal point (OP), and results.^a

Run	Variables and levels			Results		
	Gradient time (min) (x_1)	% of EtOH in B ^b (v/v) (x_2)	Flow rate (ml/min) (x_3)	GCFR	HPLC-EAT	GCFR HPLC-EAT
1	-1 (24)	-1 (20)	-1 (1.4)	145.0	48.3	3.0
2	+1 (36)	-1 (20)	-1 (1.4)	271.6	72.4	3.8
3	-1 (24)	+1 (80)	-1 (1.4)	191.0	34.4	5.6
4	+1 (36)	+1 (80)	-1 (1.4)	362.6	51.5	7.0
5	-1 (24)	-1 (20)	+1 (2.6)	91.4	89.6	1.0
6	+1 (36)	-1 (20)	+1 (2.6)	222.1	134.4	1.7
7	-1 (24)	+1 (80)	+1 (2.6)	204.9	63.8	3.2
8	+1 (36)	+1 (80)	+1 (2.6)	313.7	95.7	3.3
9	-1.683 (20)	0 (50)	0 (2)	84.9	49.2	1.7
10	+1.683 (40)	0 (50)	0 (2)	320.3	98.3	3.3
11	0 (30)	-1.683 (0)	0 (2)	145.4	94.4	1.5
12	0 (30)	+1.683 (100)	0 (2)	310.0	53.1	5.8
13	0 (30)	0 (50)	-1.683 (1)	237.4	36.9	6.4
14	0 (30)	0 (50)	+1.683 (3)	283.2	110.6	2.6
15	0 (30)	0 (50)	0 (2)	212.4	73.8	2.9
16	0 (30)	0 (50)	0 (2)	229.6	73.8	3.1
17	0 (30)	0 (50)	0 (2)	252.1	73.8	3.4
18	0 (30)	0 (50)	0 (2)	252.1	73.8	3.4
19	0 (30)	0 (50)	0 (2)	242.8	73.8	3.3
OP	+1.683 (40)	+1.683 (100)	+1.683 (3)	344.5 ± 30.2	106.2	3.2

^a Codified values are given without brackets, whereas the corresponding real values are indicated in brackets.

^b B is MeCN or EtOH in binary mobile phases (MP) or mixture of them in ternary mobile phase.

Solid phase extraction (SPE)

The stationary phase (Agilent Bond Elut C18, Vac 6 cm³, 500 mg) was first activated with 5.1 ml of EtOH and then equilibrated with 5.1 ml of H₂O–EtOH 15:85 (v/v). The column was loaded with ca. 50 mg of the dry extract and eluted with 5.1 ml of the H₂O–EtOH 15:85 (v/v) (Funari et al., 2014a). The dried eluate (43.8 mg) was solubilized in H₂O–EtOH 3:7 (v/v) to yield a final concentration of 20 mg/ml.

High performance liquid chromatography (HPLC)

HPLC analyses were performed using a HPLC-UV/PAD instrument (Shimadzu, Japan), equipped with a degasser (DGU-20A₃), two pumps (LC 20AT), an auto-sampler (SIL-20A), an UV photodiode array detector (SPD-M20A), and an oven (CTO-20A). Separations were achieved using two coupled C-18 monolithic columns (Phenomenex Onix Monolithics C-18, 100 × 4.6 mm each). Mobile phases were composed by an 0.2% (v/v) acetic acid aqueous solution (A) and pure EtOH, pure MeCN, or their mixtures (B) (Table 1). Gradient elution was performed from 3 to 85% B in 20, 24, 36, or 40 min (Table 1). Flow rates varied from 1 to 3 ml/min (Table 1). An aliquot of 15 µl of the 20 mg/ml solution mentioned in subsection 2.4 was injected in each analysis. Chromatograms were monitored at λ = 254 nm. A peak was considered any manifestations with a minimal S/N of 3. Chromatographic data were accessed using the LC Solutions software (Shimadzu, Japan). Statistical analyses were performed using Matlab 2011a (Mathworks, USA), OriginPro 8 (OriginLab, USA) and Excel 2007 (Microsoft, USA) software.

Results and discussion

An extract obtained from aerial parts of *L. sidoides* was selected as the complex sample analyzed in this work. Especially in the North-eastern Brazil, extracts from the aerial parts of this species are widely used in traditional medicine as well as in governmental programs for primary health care (Almeida et al., 2010; Lima et al., 2015). They are directly applied to treat skin wounds and mouth infections as well as to prevent and treat fungal infections in other

parts of the body when added to liquid soap formulations (Almeida et al., 2010; Lima et al., 2015). Flavonoids, lignans, and quinones have been identified in such extracts (Almeida et al., 2010; Lima et al., 2015). More recently, *L. sidoides* was included in the Brazilian Health Ministry's priority list for phytotherapeutic product development (Ministério da Saúde, 2008).

In the search for an optimal separation of an ethanol extract from aerial parts of *L. sidoides*, the influences of the gradient time (x_1), percentage of EtOH in B (x_2), and flow rate (x_3) variables were evaluated in a Central Composite Design (CCD) (Table 1). The variable x_3 , which in the range evaluated, could be pure EtOH, pure MeCN, or a mixture of them. This was a practical way to estimate the importance of the organic solvent identity in the separation of this complex sample. Due to the complexity of the sample selected here, two 100 × 4.6 mm monolithic columns were coupled in series, thus doubling the theoretical plates compare to a single monolithic column.

A function called *Green Chromatographic Fingerprint Response* (GCFR) was used as the response to be maximized (Eq. (1)) (Funari et al., 2014a):

$$GCFR = n \left(\frac{FP}{MP} \right) \left(\frac{n}{t} \right) \quad (1)$$

where n is the total number of peaks of a chromatogram, t is the total run time, FP is the number of peaks in the half of the chromatogram with fewer peaks, and MP is the number of peaks in the other half of the chromatogram. More information about this response function is available elsewhere (Funari et al., 2014a).

The CCD and its outcomes are shown in Table 1. Fig. 1 contains the chromatograms of three selected experiments.

The best result was observed with experiment 4, with GCFR of 362.6 (Table 1). Run 4 showed the best trade-off between the terms of the Eq. (1). It presented the second highest number of peaks (122, Term 2 of Eq. (1)), the second most homogenous distribution of peaks between the two halves of the chromatogram (0.88, Term 2 of Eq. (1)), and the fourth greater number of peaks per minute (3.4, Term 3 of Eq. (1)) of the series. On the other hand, the second-best score was observed for run 10 (320.3, Table 1). Although this condition led to the highest number of peaks (131), it presented

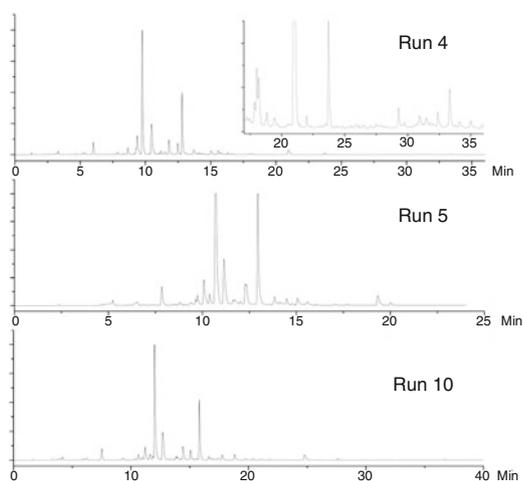


Fig. 1. HPLC-UV chromatograms of the ethanol extract of *Lippia sidoides* at $\lambda = 254$ nm for experiments 4, 5, and 10. Columns: two coupled Onyx Monolithic C-18, 100×4.6 mm each. Mobile phase components: 0.2% HOAc in water (A) and MeCN:EtOH at 2:8, 8:2, and 1:1 (vol/vol) (B) for runs 4, 5, and 10, respectively. Gradient elution: 3–85% B in 36, 24, and 40 min for runs 4, 5, and 10, respectively. Flow rates: 1.4, 2.6, and 2 ml/min for runs 4, 5, and 10, respectively. Sample injection: $15 \mu\text{l}$ of a 20 mg/ml solution.

Table 2
Analysis of variance (ANOVA) for the central composite design.

Sources of variation	Degrees of freedom	Sum of the squares	Mean of the squares	F ratio
Total	18	102,067.9	5670.4	MQ_R/MQ_f 62.8
Regression (R)	3	94,539.5	31,513.2	MQ_{pe}/MQ_f 1.7
Residual (r)	15	7528.4	501.9	
Pure error (pe)	4	2918.4	729.6	
Lack of fit (lf)	11	4609.9	419.1	

only the sixth and seventh values for terms 2 and 3 of Eq. (1) (3.3 and 0.75, respectively).

From the set of experiments indicated in Table 1, the mathematical model (with 95% confidence) was found to be as follows:

$$GCRF = 239.98 + 68.34x_1 + 45.31x_2 - 13.70x_1^2 \quad (2)$$

Table 2 presents the analysis of variance (ANOVA) for the central composite design employed here. A determination coefficient of 0.926 was obtained, with 92.6% of explained variance and 97.1% of maximum explainable variance (since pure error cannot be modeled). The F ratio shows that the model has high power prediction, because the F ratio for MQ_R/MQ_f is around 20 times greater than the F critical ($F_{3,15} 95\%$ 3.29). This indicates that the variance from the regression was significantly higher than the one from the residuals. In addition, the model did not present lack of fit, because there was no significant difference among the variances from lack of fit and pure error. The ratio MQ_{pe}/MQ_f has a value of 1.7, which is lower than the F critical ($F_{4,11} 95\%$ 3.36). This showed that the variance of the pure error is equivalent to the variance from the lack of fit.

Thus, the mathematical model (Eq. (2)) was used to predict the chromatographic condition which could lead to the highest GCFR. This condition should be reached when variables x_1 and x_2 were in their highest levels of the experimental design (+1.68). In other words, gradient time and % of EtOH in B should be set at 40 min and 100%, respectively. It is important to highlight that factor x_3 was not significant when this response (GCFR) was maximized. This meant that any value between 1 and 3 ml/min could be set for x_3 with no impact on GCFR. Thus, a flow rate of 3 ml/min was selected and the predicted optimal point was experimentally checked, leading to a GCFR score of 344.5 ± 30.2 ($n = 6$). While this value was statistically

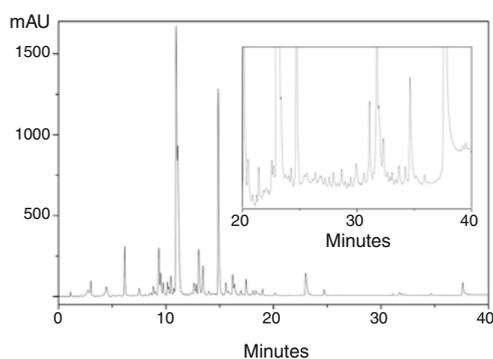


Fig. 2. HPLC-UV chromatogram of the ethanol extract of *Lippia sidoides* at $\lambda = 254$ nm for the predicted optimum point. Columns: two coupled Onyx Monolithic C-18, 100×4.6 mm each. Mobile phase components: 0.2% HOAc in water and EtOH. Gradient elution: from 3 to 85% of EtOH in 40 min. Flow rate: 3 ml/min. Sample injection: $15 \mu\text{l}$ of a 20 mg/ml solution.

similar to that obtained for the best condition in the original CCD (run 4, Table 1), it was also similar to the theoretically predicted value (392.3). This evidenced that the model was able to understand the influences of the variables on the process of separation of the ethanol extract of *L. sidoides*. A representative chromatogram acquired using the predicted optimum point is shown in Fig. 2.

The smaller peaks between 24 and 38 min (see enlargement of Figs. 1 and 2) were also considered in our response function because they were important to obtain a fingerprint of the sample. That is because a fingerprint is a multianalyte approach and as such it should provide as much chemical information about the sample as possible. Even compounds available in small amounts in plant extracts could have an desirable or undesirable impact on a given biological activity (Tistaert et al., 2011). A proper chromatographic fingerprint is recommended by the World Health Organization (WHO) for quality control of plant based medication and their derived products, such as standardized extracts, nutraceuticals, phytotherapeutics, among others (Tistaert et al., 2011; Funari et al., 2015).

The SPE step carried out here with a C18 stationary phase could potentially eliminate non-polar compounds from the original extract, with implication on the HPLC-UV fingerprint. On the other hand, if no SPE was employed, the same non-polar compounds would interact irreversibly with the HPLC column. As a result, they would not contribute to the fingerprint while reducing the life time of the HPLC column. Therefore, a conservative approach was adopted. The H_2O –EtOH 15:85 (v/v) mobile phase was employed in the SPE step, since it has similar elutropic strength of pure MeOH on C18, while being greener. This mobile phase would be able to elute from the SPE cartridge all compounds that could be eluted during the subsequent HPLC analyses. Although not infallible, this was a practical way to protect the column while minimizing a loss of signals for the desired fingerprint.

Regarding the backpressure limitation, even in this most critical condition (pure EtOH as solvent B, two coupled monolithic columns, and 3 ml/min) at any time during the run the system backpressure surpassed 250 bar. This finding is consistent with that of Destandau and Lesellier (2008), who reported a maximum backpressure of 100 bar when H_2O :EtOH mobile phases at 1 ml/min were used together with a single monolith column with the same geometry of those employed here (Destandau and Lesellier, 2008).

Although the primary goal of this work was not to find the greenest process of separation, but to check whether there were limitations on employing the viscous H_2O :EtOH mobile phase together with coupled monolith columns to separate complex samples when a standard HPLC is available, a more involved comparison of the experiments performed here was also carried out. A simpli-

fication of the so called *Comprehensive Metric to Compare Liquid Chromatography Methods* (CM) (Funari et al., 2014b) was employed as follows:

$$CM = \frac{GCFR}{HPLC - EAT} \quad (3)$$

The numerator of Eq. (3) is the same as the whole Eq. (1), while its denominator is the HPLC-Environmental Assessment Tool (HPLC-EAT), which was first developed by Gaber et al. (2011) to measure the greenness of HPLC methods. It takes into account parameters related to the Safety, Health, and Environment (SHE) and life-cycle-assessment (LCA) of solvents involved in an analysis. It is easy to calculate (a free software is provided by the authors) and allows the discernment between LC methods that might not be recognized by metrics that were not developed specifically to HPLC applications (Funari et al., 2014b). However, it lacks information on the performance of separation, which is why HPLC-EAT was combined with GCFR in this work. Thus, the final score provided by Eq. (3) considers environmental parameters given by HPLC-EAT and parameters of separation given by GCFR. Results are shown in Table 1.

This metric (Eq. (3)) evidenced that the best trade-off was obtained with run 4, which was better than that obtained with any other condition (Table 1), including that predicted by the mathematical model (Eq. (2)). This finding illustrates the importance of comparing processes in a heuristic way. If only the “greenness” of the solvents used here were taken into consideration, one would conclude that the optimized method predicted by the mathematical model (Eq. (2)) was better than run 4. That was because while the former employed only the greener EtOH as B, the latter employed MeCN:EtOH 2:8 (vol/vol), which meant that 20% of the organic modifier was the problematic acetonitrile. However, the proper metric used here (Eq. (3)) found that the opposite was true since the higher amount of organic modifier (EtOH) used to run the predicted optimum point method had greater impact than the use of 20% MeCN in the organic modifier as in run 4. This case illustrates the definition of Dr Philip Jessop, for who the greenest solvent is that which leads to the greenest process.

Conclusion

This work presented a strategy to replace the problematic acetonitrile with the more viscous EtOH in a HPLC system that can accommodate backpressures of no more than ca. 300 bar. The study found that there is no limitation for such replacement when monolith columns were used. Flow rates of up to 3 ml/min were accommodated with two coupled monolithic columns (100 × 4.6 mm) without exceeding 250 bar. The findings presented here also showed the need for comprehensive metrics when comparing the greenness of two or more processes of separation. Safety, health, and environmental parameters should be considered both qualitative and quantitatively together with parameters of separation as the CM employed here. A comparison based only on the score given by solvent selection guides is not enough as exemplified here when run 4 and the optimized method were compared, evidencing the advantage of using the former even if a small percentage of MeCN was added to the organic modifier. The new generation of monolithic columns available on the market today could help replace traditional harmful solvents with the more viscous EtOH without prejudicing the separation required in practical applications even when a less potent HPLC is the only instrument available to the analyst. These columns exhibit efficiencies comparable to those observed for sub-2-μm fully porous or sub-3-μm core-shell packed columns while generating less resistance to the mobile phase flow. The use of smaller monolith columns would also be compatible with such instruments, while reducing the consumption of organic solvent. These findings rein-

forced that no state-of-the-art instruments are needed to shift from traditional harmful solvents to greener ones, but only a change in researchers' approach toward sustainability. We believe that this shift should be led by people involved in natural products investigation, especially those working with medicinal plants and derived products. It makes sense to be concerned with the health of the environment in which samples are extracted from as well as with the health of researchers and apprentices involved in the research about plants and derived products to improve the well-being of everyone.

Authors' contributions

CSF performed all the experimental work, analyzed the data and wrote the manuscript. RLC and AJC contributed to experimental design, data analysis, and drafting of the manuscript. All the authors read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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