Development and optimization of a HILIC-UPLC-ELSD method for simultaneous determination of eleven polyols from glycerin bioconversion processes

Pedro Ribeiro Fontes[1], Patrícia Pinto Kalil Goncalves Costa[2], Kaian Amorim Teles[3], Carolina Madalozzo Poletto[4], Mônica Caramez Triches Damaso[5], Clenilson Martins Rodrigues[6]

Abstract

To prevent the constant greenhouse emissions caused by fossil fuels, renewable energy sources are becoming of utmost relevance to diversify worldwide energy matrix. Alternative energy sources, such as biofuels and biomass are nowadays focus of several studies due to their great potential as matrices to bioenergy that can be used in complement to fossil fuels. Brazil has a huge potential for production of biofuels (e.g., 1st and 2nd generation ethanol, biodiesel) considering that the country has vegetal feedstocks and agricultural residues highly available, once it is one of the greatest agricultural commodities producer in the world. Biodiesel production generates considerable amounts of glycerol as a by-product and it is necessary to overcome this problem with feasible solutions. Bioconversion in biorefinery-based systems is one of them and monitoring the production of building blocks or value-added compounds from the bioconversion of renewable sources like glycerol is essential to assure effectiveness of such systems. In this work, we aimed to develop and perform an initial evaluation of some analytical parameters of a HILIC-UPLC-ELSD method for determination of eleven different polyols as products of glycerin bioconversion processes based on the biorefinery concept. The eleven target compounds were successfully separated and two methods were developed to correctly identify and quantify all of them. Preliminary evaluation of analytical parameters revealed that

[1] Farmacêutico, mestre em Ciências Farmacêuticas, Universidade de Brasília, pedro.r.fontes@colaborador.embrapa.br
[2] Química, mestre em Química Orgânica, analista da Embrapa Agroenergia, patricia.costa@embrapa.br
[3] Farmacêutico, mestrando em Patologia Molecular, Universidade de Brasília, kaian.teles@gmail.com
[4] Engenheira de alimentos, mestre em Microbiologia Agrícola e do Ambiente, analista da Embrapa Agroenergia, carolina.poletto@embrapa.br
[5] Engenheira química, doutora em Tecnologia de Processos Químicos e Bioquímicos, pesquisadora da Embrapa Agroenergia, monica.damaso@embrapa.br
[6] Químico, doutor em Química, pesquisador da Embrapa Agroenergia, clenilson.rodrigues@embrapa.br

both methods presented good linearity ($R^2 \geq 0.99$) and sensitivity (LOQ ≤ 100 ng of analyte in the column). Experiments are currently being conducted to perform full method validation and to evaluate polyols production by biotransformation of glycerol.

Introduction

Diversification of energy matrix to include alternatives to fossil fuels is becoming a very important issue in several countries. Biomass and its derivatives are among the most promising options to the evidently limited fossil fuels. Examples to be cited include first and second generation ethanol, produced from vegetal feedstocks, and biodiesel, which can be produced either from vegetal oils or animal fats (DA SILVA et al., 2009).

Commercially, biodiesel is produced by a transesterification reaction between a source of triglycerides and an alcohol (methanol) in the presence of a catalyst. This reaction produces the fatty acid methyl esters (FAME) and glycerol as by-product (LEONETI et al., 2012). Since by-product glycerol is accumulating and neither sustainable nor rentable destination has been developed yet, searching for reuse solutions has become of utmost importance.

Polyols can be produced from the reduction of aldehyde or ketone functions from carbohydrates (ALMEIDA et al., 2012). They have a variety of uses, such as food additives, pharmaceutical adjuvants and intermediates in chemical synthesis. Polyols such as xylitol, mannitol and erythritol can also be obtained by biorefinery-based bioprocesses, which reuse industrial or agricultural wastes as carbon source to production of other value-added compounds by microorganisms.

It is important to consider that biochemical machinery of microorganisms is very complex and some biosynthesis routes may not be specific to produce only one type of polyol isomer. This complexity becomes greater with higher number of possible compounds produced by a specific microorganism. Given that, it is imperative to develop analytical tools to accurately identify and quantify such compounds, considering that their production would be very advantageous to both economic and environmental aspects. Polar compounds like polyols are generally evaluated using Hydrophilic interaction liquid chromatography (HILIC) as a separation mode. In this work is presented the development and an initial evaluation of some analytical validation parameters of a HILIC-UPLC-ELSD method

for determination of eleven different polyols as products of glycerin bioconversion processes.

Material and methods

Analytical standards and chemicals

Polyol standards (glycerol, two $C_4H_{10}O_4$ isomers [C4a and C4b], three $C_5H_{12}O_5$ isomers [C5a, C5b and C5c], four $C_6H_{14}O_6$ isomers [C6a, C6b, C6c and C6d] and one $C_7H_{16}O_7$ isomer [C7a] — Table 1) and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St Louis, MO, USA). HPLC-grade acetonitrile was purchased from Tedia® (Fairfield, OH, USA). Ultrapure water (18 MΩ cm) was obtained from a Direct 16 Milli-Q purification system (Millipore Co., Bedford, MA, USA).

Table 1. Polyol's isomers labeled according to the number of carbon atoms in the structure. Compound's identification is under protected rights in this work.

C3	C4	C5	C6	C7
Glycerol	C4a	C5a	C6a	C7a
	C4b	C5b	C6b	
		C5c	C6c	
			C6d	

Instruments and method validation

This work was started with the instrumental parameters previously described by Fontes et al. (2016) and this configuration (

Table **2**, a) was used during the optimization of the HILIC-UPLC-ELSD gradient method. An Acquity UPLC H-class system (Waters®, Milford, MA, USA) and an evaporative light scattering detector (ELSD, Waters®) controlled by Empower PRO 3 software was used to perform method development. Separation was achieved on gradient mode (Table 2, **Erro! Fonte de referência não encontrada.**b and c) using a UPLC BEH-Amide column (2.1 x 150 mm, 1.7 µm; Waters®) equipped with its respective pre-column.

Table 2. Instrumental parameter set for polyols determination. [a] previous method described by Fontes *et al.* (2016); [b] LC condition for C3, C4a, C4b, C5a, C5b, C5c, C6a, C6b and C7 separation. [c] LC condition for C6c and C6d separation.

System	Parameters	Initial condition[a]	Method 1[b]	Method 2[c]
ELSD	Nebulizer gas pressure (psi)	25.0	40.0	40.0
	Photomultiplier gain	200	200	200
	Drift tube temperature (°C)	34.0	50.0	50.0
	Nebulizer mode	Cooling	Cooling	Cooling
UPLC	Mobile phase flow (mL/min)	0.3	0.3	0.3
	Column temperature (°C)	65.0	30.0	73.0
	Injection volume (µL)	2.0	0.5	0.5
	Elution mode	Isocratic	Gradient	Gradient

The gradient was composed of 0.05% TFA in ultrapure water (A) and 0.05% TFA in acetonitrile (B), running as follows (percentages of A/B): 5/95 with isocratic elution (zero to 9.00 min); 15/85 to 34/66 with linear gradient elution (9.00 to 15.00 min); 5/95 with isocratic elution (15.01 to 20.00) for column re-equilibration. Total run time was 20.00 minutes.

Limits of detection (LOD) and quantification (LOQ) were determined by the signal-to-noise (S/N) method (ICH …, 2005): the concentration at which the method returned S/N ≥ 3 and S/N ≥ 10, respectively. All analytical curves were determined in triplicate, and LOQ values were chosen as starting concentrations for the curves. Linearity was evaluated by linear least squares regression method and regression lines were subjected to Analysis of Variance (ANOVA; $\alpha = 0.05$) to evaluate statistical significance. Model fitting was determined by visual inspection of regression residuals scatter plot.

Results and discussion

The isocratic method that was before applied only to 3 analytes (FONTES et al., 2016) was successfully adapted to the present scenario, which demands the separation of eleven different polyols. In the first condition (Table 2b), nine

polyols were successfully separated within 20 min. However, two C6 compounds (C6a and C6b) co-eluted at 11.12 min (Figure 1, A). For this reason, a new attempt to separate these analytes was performed changing the column temperature to higher values (second condition, Table 2c). The co-eluted C6 polyols were partially separated at 73°C and this condition allows the differentiation between the both C6 isomers as well as the quantification of them (Figure 1, B). To cover the eleven analytes, these two methods were adopted: Method 1 to all polyols with the exception of C6a and C6b; for these two, Method 2 was adopted.

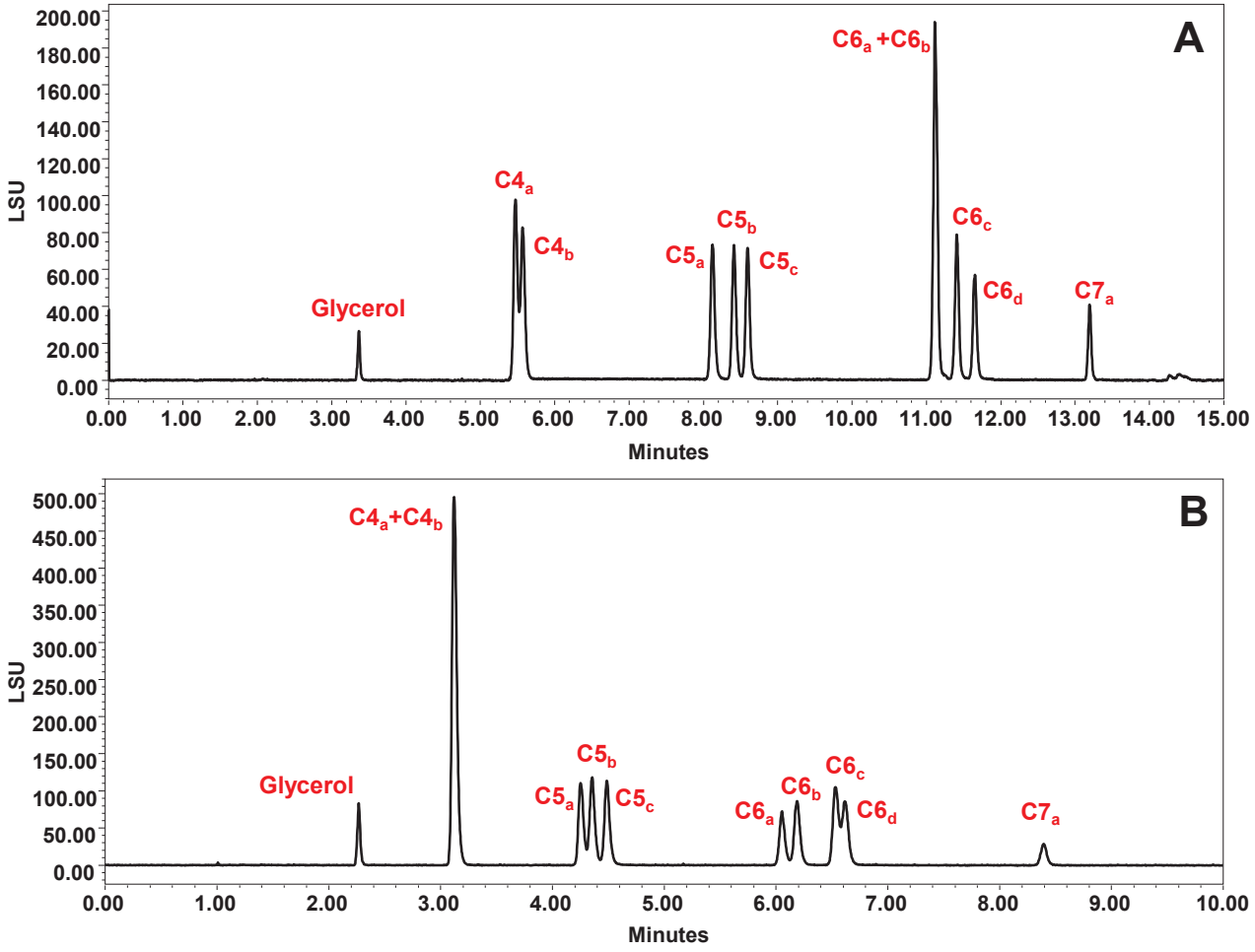

Figure 1. Chromatograms of a mix (11 polyols) standard solution at 400 µg/mL running as described in Table 2. (A) 15.00 minutes chromatogram cut running at 30°C and (B) 10.00 minutes chromatogram cut running at 73°C. LSU: arbitrary light scattering units.

The LOD and LOQ were determined to the eleven compounds and are represented in Table 3, in terms of analyte mass injected in the column.

Table 3. Limits of detection (LOD) and quantification (LOQ) and linearity coefficient of determination for each polyol studied.

Analyte	R^2	LOD	LOQ	Analyte	R^2	LOD	LOQ
Glycerol	0.9925	< 1 µg	1.5 µg	C6a	0.9998	50 ng	75 ng
C4a	0.999	50 ng	75 ng	C6b	0.9941	62.5 ng	0.1 µg
C4b	0.9995	50 ng	75 ng	C6c	0.9999	50 ng	75 ng
C5a	0.9998	40 ng	62.5 ng	C6d	0.9998	45 ng	62.5 ng
C5b	0.9999	50 ng	75 ng	C7a	0.9999	50 ng	75 ng
C5c	0.9997	40 ng	62.5 ng	----	----	----	----

Linearity data analysis was performed with both mass and peak area data transformed to the logarithm form, given that the mathematical relation between a compound mass and its ELSD response is not linear, but exponential (FONTES et al., 2016). Regression lines to all polyols studied in this work presented a coefficient of determination (R^2) ≥ 0.99 (Table 3). Regression models were also statistically significant and showed to be fitted to the data. Also, all angular coefficients were significantly different from zero.

Conclusions

The methods presented in this work showed to be suitable for the purpose they were developed, being capable to separate the eleven analytes considered in the study. The separation achieved makes possible the correct identification and quantification of all polyols' isomers within 15.00 minutes in Method 1 and 10.00 minutes in Method 2 (

Table **2**; Figure 1). The HILIC-UPLC-ELSD methods showed to be linear in the range studied with all R^2 ≥ 0.99. It also presented good sensitivity with all LOQs ≤ 100 ng of analytes mass on the column, with the exception of glycerol. Nevertheless, the higher LOQ observed for glycerol does not represent a loss on method quality, since this compound is expected to be found at high concentrations in real samples compared to the target polyols. The developed methods remain to be fully validated and applied to real samples of bioconverted glycerol. Experiments are currently being conducted to evaluate these analytical aspects.

Financial support

The authors gratefully acknowledge the financial support of the CNPq (Proc. N°. 404854/2013-3) and Embrapa (Proc. N°. 03.14.04.004.00.00). Thanks are also extended to CNPq for a fellowship to P.R.F and K.A.T.

References

ALMEIDA, J. R.; FAVARO, L. C.; QUIRINO, B. F. Biodiesel biorefinery: opportunities and challenges for microbial production of fuels and chemicals from glycerol waste. **Biotechnology for Biofuels,** London, v. 5, n. 48, 2012.

DA SILVA, G. P.; MACK, M.; CONTIERO, J. Glycerol: a promising and abundant carbon source for industrial microbiology. **Biotechnology Advances**, Oxford, v. 27, n. 1, p. 30-9, 2009.

FONTES, P. R.; RIBEIRO, J. A. A.; COSTA, P. P. K. G.; DAMASO, M. C. T.; GONZALEZ, W. D.; DOS SANTOS, C. M. C.; ABDELNUR, P. V.; RODRIGUES, C. M. Development and validation of a HILIC-UPLC-ELSD method based on optimized chromatographic and detection parameters for the quantification of polyols from bioconversion processes. **Analytical Methods**, Cambs, v. 8, n. 9, p. 2048-2057, 2016.

ICH Harmonised Tripartite Guideline: validation of analytical procedures: text and methodology Q2(R1). [S.l.]: ICH, 2005. 13 p. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use.

LEONETI, A. B.; ARAGÃO-LEONETI, V.; DE OLIVEIRA, S. V. W. B. Glycerol as a by-product of biodiesel production in Brazil: Alternatives for the use of unrefined glycerol. **Renewable Energy**, Oxford, v. 45, p. 138-145, 2012.