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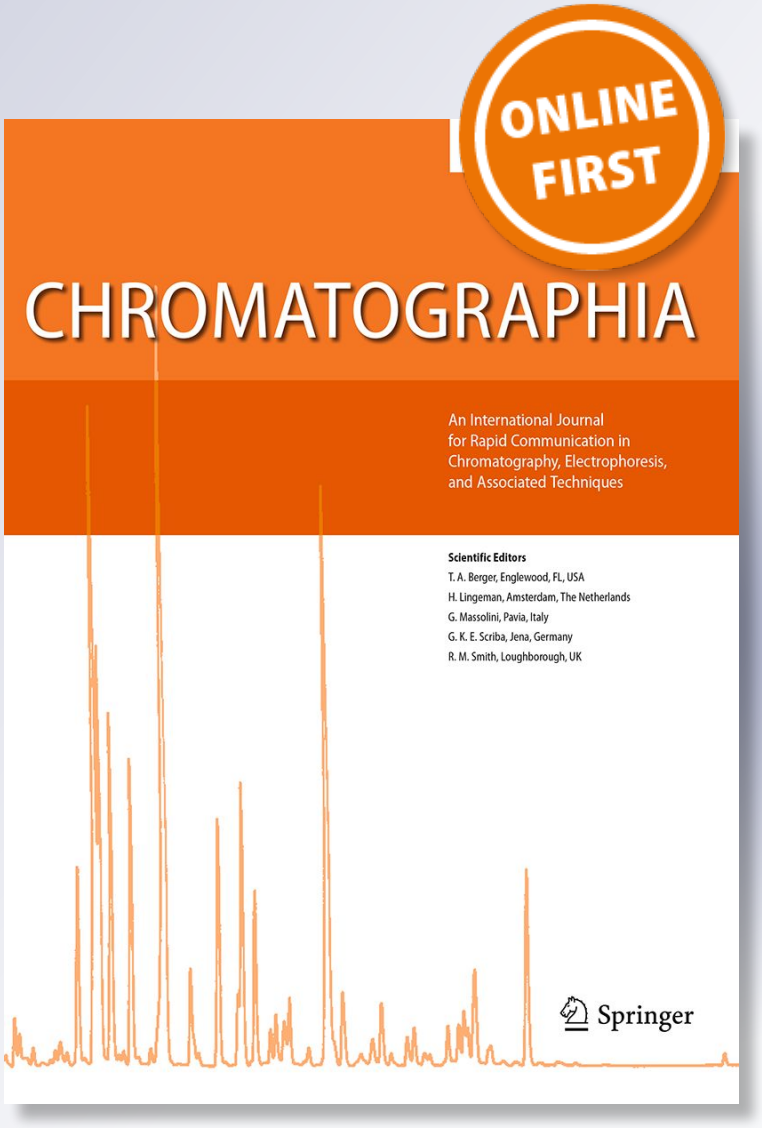

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SHORT COMMUNICATION

Optimization and Validation of a GC–FID Method for Quantitative Determination of 1,3-Propanediol in Bacterial Culture Aqueous Supernatants Containing Glycerol

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Abstract 1,3-Propanediol (1,3-PDO) is an important chemical widely used in polymer production. This compound has been traditionally obtained by chemical synthesis, but it can also be obtained from bacterial fermentation using glycerol as renewable carbon source. Since 1,3-PDO and glycerol have a similar chromatographic behavior, it is not easy to determine both compounds efficiently in short runs. In this work, we optimized a rapid and simple capillary GC–FID method for the determination of 1,3-PDO and glycerol directly in bacterial culture supernatants. Good peak shapes and high resolution were obtained with no significant tailing. The method was validated in terms of specificity, linearity, precision, accuracy, limit of detection, limit of quantitation, robustness and stability according to USP and ICH guidelines. The method can be applied for the determination of 1,3-PDO and glycerol in different media in less than 5 min with high precision, reproducibility and selectivity. No time-consuming extraction or concentration steps and no significant matrix effects make this method outstanding for glycols determination.

Keywords 1,3-Propanediol · Glycerol · GC–FID · Bacterial supernatants

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Introduction

1,3-Propanediol (1,3-PDO) is a valuable chemical precursor, suitable for the production of diverse industrially relevant molecules, such as polyesters, polyethers and polyurethanes [1]. Among them, a new generation 1,3-PDO-based polymer called trimethylene terephthalate is particularly interesting due to its outstanding properties, such as high elasticity, biodegradability and chemical resistance [2]. Over 120,000 tons of 1,3-PDO were produced in 2007 [3] and the demand for PDO-based products is constantly growing [3].

This compound has been traditionally obtained from non-renewable resources in processes that are highly energy demanding and not environmentally friendly. Since 1,3-PDO could be produced by microbial conversion, great efforts have been made to improve processes involving different microorganisms that can produce 1,3-PDO from glycerol [4].

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Accurate determination of 1,3-PDO is a key task for both industry and research. In the past decades, it has been analyzed by GC using packed columns [5, 6] in spite of its poor resolution and long run times. Current methods to measure this compound involve HPLC–RID [7, 8], in spite of low sensitivity and temperature-dependence of this universal detector. Only few reports include GC determinations for 1,3-PDO, and most of them do not determine glycols in complex matrixes, such as those of biological origin [9]. These methods cannot be applied directly to aqueous solutions, and require time-consuming extraction steps which may affect analyte recovery [10–12].

In this work, we propose a simple and rapid capillary GC–FID method for the determination of 1,3-PDO in aqueous supernatants from bacterial cultures without extraction steps. Easy sample preparation and the simultaneous measurement of glycerol (carbon source) make this method a suitable tool for monitoring microbial conversions of glycerol to 1,3-PDO. The method was validated and optimized for very short time analysis and excellent resolution of both chemicals.

Materials and Methods

Chemicals

1,3-PDO from Merck (Darmstadt, Germany), ethanol from Sintorgan (Buenos Aires, Argentina) and glycerol obtained from Sigma–Aldrich (Darmstadt, Germany) were used. All reagents were HPLC grade.

Stock Solutions

Stock solutions for both analytes were prepared by dilution of pure standards in M9 mineral medium. Concentrations were 100 and 200 g L^{-1} for 1,3-PDO and glycerol, respectively.

Sample Preparation

Standards and stock solutions were prepared in M9 medium. For the chromatographic determination, 250 µL of standards were diluted in 750 µL of ethanol (used as chromatographic solvent), vortexed for 1 min and centrifuged at 9000 rpm for 3 min at 4 °C for salt precipitation. Biological samples were microfiltered through 0.22 µm nylon membranes (MSI, USA), stored at −20 °C, thawed at room temperature if necessary, and finally diluted in ethanol and centrifuged as described above for the analysis of standards.

Chromatographic Conditions

1,3-PDO and glycerol were measured using an Agilent 7820A GC–FID with an automatic liquid sampler ALS 7693. The separation was conducted on a HP-INNO-WAX capillary column (30 m, 0.25 µm film thickness and 0.25 mm ID). The GC oven was initially heated at 185 °C for 3 min, then to 220 °C at 40 °C/min, and held for 1 min. The injector and FID temperatures were set at 290 and 300 °C, respectively. Nitrogen was used as carrier gas at a column flow of 2.5 mL/min. The injection volume was 2 µL with a 30:1 split ratio.

Bacterial Strains and Growth Conditions

Bacterial cultures of *Escherichia coli* BW25113 carrying the genes for the synthesis of 1,3-PDO from *Klebsiella pneumoniae* were grown at 37 °C and 200 rpm in M9 medium containing per Liter: Na_2HPO_4 6 g, KH_2PO_4 3 g, NaCl 0.5 g, NH_4Cl 1 g, $Fe(C_6H_5O_7)$ 0.06 g, $MgSO_4$ 0.12 g, $CaCl_2$ 0.011 g and glycerol 10 g as the sole carbon source.

Culture media were supplemented with cobalamin (0.002 g L^{-1} final concentration) and kanamycin (1 mM) for plasmid maintenance. Culture media used for selectivity assays were Lysogeny Broth (LB) containing per Liter: 4 g L^{-1} of tryptone, 4 g L^{-1} of NaCl and 2 g L^{-1} of yeast extract; Nutrient Broth (NB) from Merck and TSD medium containing per Liter: 1.85 g of $(NH_4)_2SO_4$, 0.05 g of $FeSO_4.7H_2O$, 1.0 g of KH_2PO_4, 1.0 g of $MgSO_4$, 0.1 g of $CaCl_2.2H_2O$, 2 g of trisodium citrate, 0.5 g of yeast extract, 0.002 g of *p*-amino benzoic acid, 0.002 g of thiamine–HCl, 0.01 mg of vitamin B12, 0.12 g of methionine, 1 g of L-cysteine–HCl, and 0.002 g of resazurin [13].

Validation Parameters

The linearity was evaluated by five injections at ten concentration levels. The ranges tested were 0.01–20 g L^{-1} for 1,3-PDO and 0.025–30 g L^{-1} for glycerol.

Limit of detection (LOD) and limit of quantification (LOQ) were calculated as the concentrations which generated peak heights three and ten times higher than the noise level, respectively.

Precision was tested at three levels: repeatability, intermediate precision and reproducibility. Repeatability was evaluated by injecting 1,3-PDO (0.1–20 g L^{-1}) or glycerol (0.5–20 g L^{-1}) for six consecutive times from the same sample in three concentration levels. Intermediate precision (1 g L^{-1} of 1,3-PDO and 10 g L^{-1} of glycerol) was tested by injecting samples prepared from the same stock solution in six consecutive days. Reproducibility (0.5–10 g L^{-1} of

1,3-PDO and 4–20 g L^{-1} of glycerol) was determined by injecting six samples which were prepared from independent stock solutions.

Accuracy was assessed on a blank sample matrix (M9 medium) spiked with three known amounts of 1,3-PDO or glycerol included in the working range, but different from those used for the calibration curve (0.3, 1.5 and 7.5 g L^{-1} for 1,3-PDO; 5, 7.5 and 25 g L^{-1} for glycerol). Six samples prepared independently were used for each level. The recovery percentages were then calculated by comparison of the concentrations predicted by the method with the known concentrations.

The stability of samples prepared by diluting in ethanol M9 medium spiked with 1,3-PDO (1 g L^{-1}) and glycerol (10 g L^{-1}) was tested by means of injections performed at six different time points (0, 1, 3, 5, and 24 h). All samples were stored at room temperature or frozen (−20 °C) in chromatography vials.

For selectivity determinations, samples were prepared by diluting stock solutions of 1,3-PDO and glycerol in LB, M9, TSD, NB and water. Final concentrations were 3 g L^{-1} of 1,3-PDO and 7.5 g L^{-1} of glycerol.

Robustness determinations were performed in samples containing 3 g L^{-1} of 1,3-PDO and 7.5 g L^{-1} of glycerol in M9 were adjusted to different pH (5, 7 and 8) using HCl (2 M) or NaOH (10 M).

Results and Discussion

Method Development

The non-polar column HP-5 was first tested and found suitable for 1,3-PDO determination. However, glycerol could not be analyzed in these conditions because of lack of linearity and a poorly defined peak. Polarity differences between glycerol and the stationary phase of this column [(5%-phenyl)-methylpolysiloxane)] might affect chromatographic analysis. For this reason, a column with a more polar stationary phase, HP-INNOWAX with a polyethylene glycol phase, was chosen. However, peak shapes and sensitivity were not as good as expected. A possible reason could be an interference of the aqueous medium in the interaction between the stationary phase and the analytes. To avoid this, ethanol was tested as a diluent, since it has been shown to be useful for the extraction of glycols [13]. This procedure has the additional advantage of reducing the amount of water input in the chromatographic system and promoting salt precipitation from the culture medium before sample injection. Addition of ethanol resulted in improving peak shapes and good linearity, allowing efficient chromatographic analysis.

Biological samples and undefined media are complex matrices containing salts, debris and cellular components such as proteins that can potentially block the needle. Several water washes after injection were used to avoid salt crystallization and efficient glycol removal. Ethanol was employed for the final washes to reduce water content and prevent syringe and column damage.

Oven temperature was optimized to reduce run times. At the beginning, slow gradients with low initial temperatures were tried to minimize bleeding and extend column lifetime. However, this approach resulted in broad peaks because of diffusion, high LODs and LOQs and bad reproducibility. Oven temperature was then set at 185 °C, close to 1,3-PDO boiling point, and since no other signals interfered with analyte peaks the temperature gradient was adjusted to 40 °C/min. This temperature program led to run times shorter than 5 min.

Validation

The method was validated according to USP and ICH guidelines [14, 15]. Resolution resulted higher than 4 including chromatograms of biological samples where no interference peaks were observed and tailing was less than 2. A typical system suitability chromatogram is shown in Fig. 1.

Microbial cultures can produce up to 100 g L^{-1} of 1,3-PDO, while initial glycerol concentrations used for this process are between 10 and 40 g L^{-1}, and can be completely consumed during growth. The linearity was evaluated at ten concentration levels to cover this broad range of concentrations, using five injections for each sample (taking into account ICH recommendations). Linearity was maintained throughout the whole range: 0.05–20 g L^{-1} for 1,3-PDO (correlation coefficient $R = 0.9999$) and 0.5–30 g L^{-1} for glycerol (correlation coefficient $R = 0.9967$) (Table S1).

Method sensitivity was assessed by LOD and LOQ. The concentrations of LOD were 0.010 g L^{-1} for 1,3-PDO and 0.025 g L^{-1} for glycerol. The LOQ were 0.02 g L^{-1} for 1,3-PDO and 0.050 g L^{-1} for glycerol. For both compounds, the method resulted in good sensitivity and low baseline noise.

The precision was thoroughly tested at three levels: repeatability, intermediate precision and reproducibility. Compound concentrations were chosen according to those expected for biological processes and are detailed in Table 1. Relative standard deviations (RSD) obtained were less than 3% in all cases in compliance with international standards.

For accuracy evaluation, recovery percentages were calculated at three concentration levels. For 1,3-PDO, the recovery was 97 ± 6% and for glycerol were 103 ± 11%.

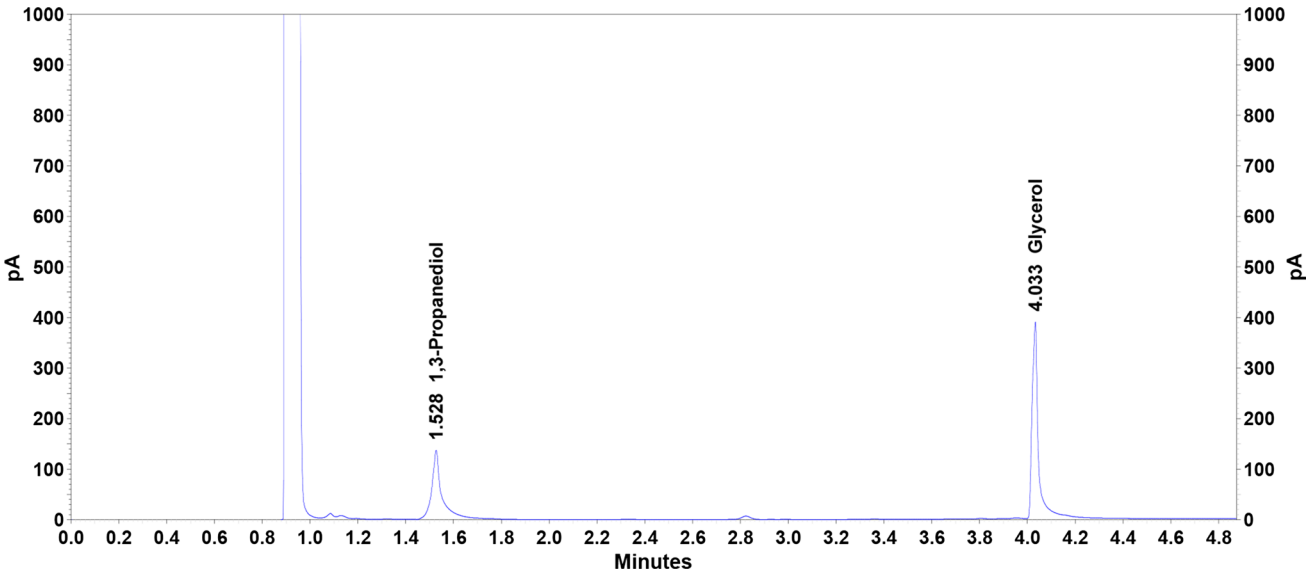

Fig. 1 Typical glycols chromatogram using the optimized method. Stock standard solutions were diluted in M9 media up to 3 and 7.5 g L^{-1} for 1,3-PDO and glycerol, respectively

Table 1 Precision analysis

	Repeatability[a]			Inter-day precision[b]	Reproducibility[c]		
	0.1 g L^{-1}	1 g L^{-1}	20 g L^{-1}	1 g L^{-1}	0.5 g L^{-1}	3 g L^{-1}	10 g L^{-1}
1,3-PDO							
1	117,983	991,456	19,337,887	1,039,576	558,819	3,158,673	9,370,859
2	125,007	983,181	19,181,450	1,076,574	528,824	3,159,375	9,458,069
3	121,515	1,038,232	20,152,012	1,084,199	565,013	3,172,272	8,783,461
4	120,371	1,037,324	20,096,406	1,028,671	564,965	3,199,421	9,518,511
5	124,917	1,043,134	19,994,328	1,060,221	575,424	3,057,287	9,238,532
6	124,302	1,057,648	19,792,606	1,070,446	564,893	3,025,642	9,311,998
Average	121,959	1,018,665	19,752,417	1,057,848	558,609	3,149,406	9,273,886
RSD (%)	2.48	2.83	2.31	2.05	2.86	2.04	2.59
	0.5 g L^{-1}	6 g L^{-1}	20 g L^{-1}	10 g L^{-1}	4 g L^{-1}	7.5 g L^{-1}	20 g L^{-1}
Glycerol							
1	176,455	3,633,387	15,236,899	6,088,541	2,566,011	4,856,872	13,499,174
2	167,757	3,540,417	14,842,806	6,601,618	2,693,641	4,893,076	13,437,765
3	163,025	3,521,908	14,351,587	5,991,205	2,719,655	4,880,771	12,593,112
4	173,012	3,460,438	14,886,584	6,357,880	2,715,942	4,939,590	13,590,933
5	166,084	3,478,043	14,874,904	6,504,141	2,761,139	4,717,173	13,332,233
6	168,377	3,475,507	15,052,918	6,483,099	2,762,198	4,594,396	12,765,286
Average	169,118	3,518,283	14,874,283	6,337,747	2,703,098	4,813,646	13,203,084
RSD (%)	2.87	1.66	1.82	3.87	2.68	2.49	2.89

[a] Six consecutive injections of each standard

[b] Samples prepared in six different days from the same stock solution

[c] Samples prepared in six different days from six different stock solutions

RSD were 0.83 ± 0.44% and 1.7 ± 0.5%, respectively. In all cases, RSDs were less than 3% in agreement with ICH guidelines. The results demonstrated that the method was suitable for the accurate quantification of 1,3-PDO and glycerol.

The stability of samples stored either at room temperature or at −20 °C was determined as indicated in materials and methods. RSD were compared against initial time for both conditions. After 24 h, RSD resulted in less than 10% for all cases indicating good sample stability. Despite high ethanol volatility, sample preparation resulted suitable for glycols analysis and no noticeable differences were observed between both temperature conditions (Table S2).

Since the method involves no extraction steps, the effect of different matrices was analyzed. Four different matrices including culture media employed for microbial cultivation (M9, LB, TSD, NB) and water, were used to evaluate the selectivity of the method for the analytes of interest. Peak areas, retention times and reproducibility were assayed comparing each matrix against M9 medium (Table 2, Figure S1). The values for coefficient of variation (CV) obtained were less than 15% showing that the matrix effects were not significant.

Since metabolites produced by different microorganisms and growing conditions can modify the final pH of the culture media, the effect of pH was evaluated. We tested two pH conditions (5 and 8) and estimated the pH effect comparing each condition against pH 7 in M9 medium (Table S3). The CVs obtained for each pH were less than 5% showing that the method could be used in a wide pH range.

Applications

1,3-PDO production and glycerol consumption were studied in cultures of *Escherichia coli* carrying the genes of *Klebsiella pneumoniae* necessary for 1,3-PDO biosynthesis and a control strain without these genes. The cultures were grown in M9 medium supplemented with 10 g L^{-1} of glycerol for 24 h. The producer strain reached up to 1 g L^{-1} of 1,3-PDO, while the remaining glycerol concentration was 2.02 g L^{-1}. When the determination was repeated using the control strain, no 1,3-PDO was detected, and the remaining glycerol concentration was 3.10 g L^{-1}. No significant differences were seen between replicates ($n = 5$), indicating good reproducibility. It should be noticed that no peaks from bacterial metabolites interfered with analytic determinations (Fig. 2a). Moreover, the resulting strain seems to be suitable for 1,3-PDO production (Fig. 2b).

Conclusions

A simple and rapid capillary GC–FID method was optimized for the simultaneous analysis of 1,3-PDO and glycerol in bacterial cultures. The method was validated according to USP and ICH guidelines. It showed high sensitivity, reproducibility, repeatability, accuracy and had

Table 2 Matrix effects1

Matrix	Mean peak area	Retention time	Repeatability (RSD%)	Reproducibility (RSD%)	Matrix factor
1,3-PDO					
M9	3,164,368	1.528	2.88	1.84	1.00
H_2O	3,300,178	1.553	0.98	3.14	1.04
TSD	3,258,966	1.552	2.89	3.72	1.03
LB	2,964,998	1.528	0.97	1.78	0.94
NB	3,731,373	1.503	3.14	3.08	1.18
Mean matrix factor					1.04
CV (%)					8.57
Glycerol					
M9	4,954,523	4.032	2.56	1.76	1.00
H_2O	5,272,928	4.048	1.34	3.67	1.06
TSD	5,757,243	4.048	2.56	2.90	1.16
LB	5,406,485	4.037	0.67	3.12	1.09
NB	6,743,953	4.023	1.01	2.34	1.36
Mean matrix factor					1.14
CV (%)					12.27

Samples containing 3 g L^{-1} of 1,3-PDO and 7.5 g L^{-1} of glycerol were prepared in each different matrix from the same stock solutions

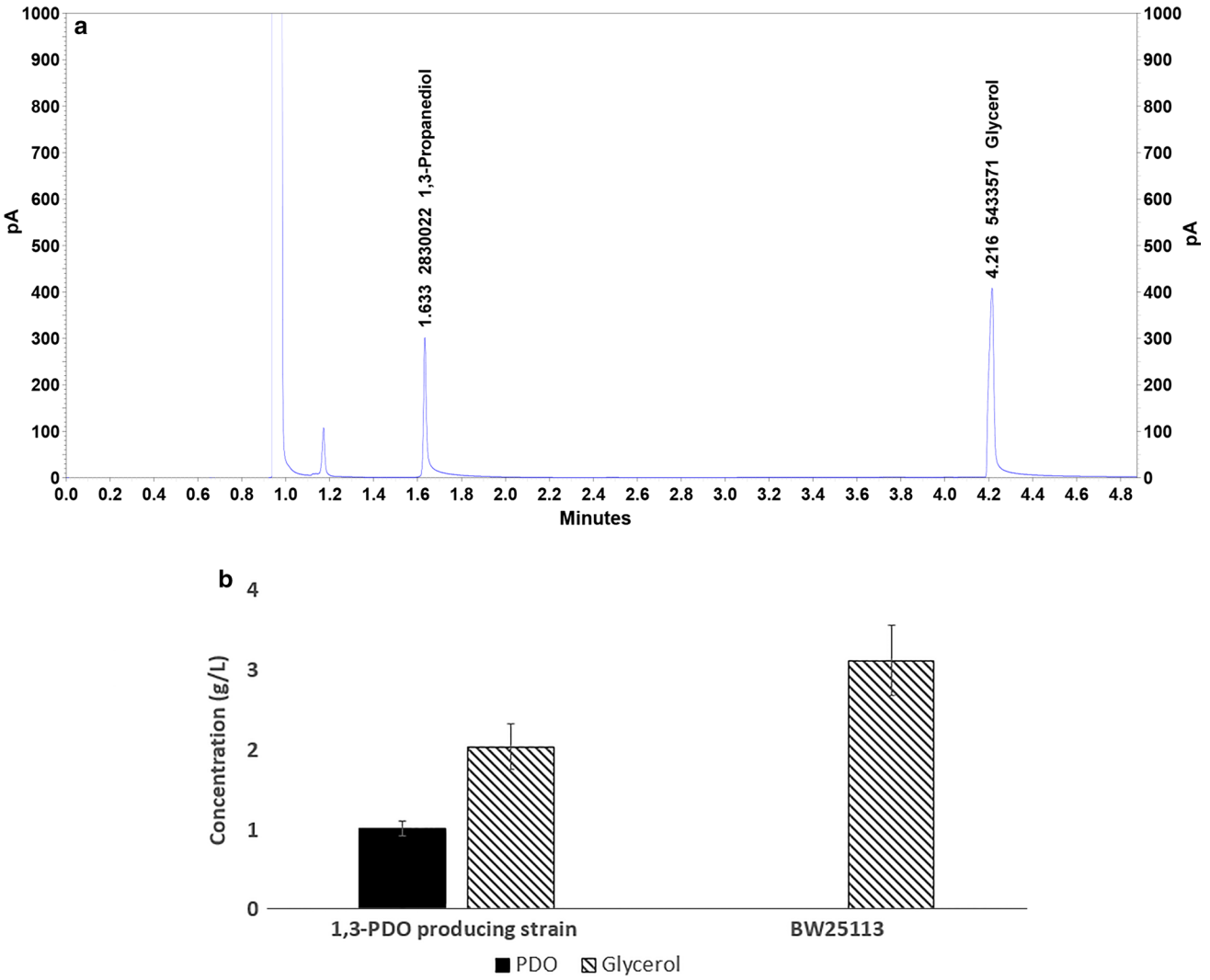

Fig. 2 **a** Typical chromatogram of a bacterial culture supernatant from *Escherichia coli* producing 1,3-PDO from glycerol as carbon source, **b** 1,3-PDO production and consumed glycerol in 1,3-PDO producing strain and in control strain. Culture was carried out in M9 mineral medium

linear behavior in the range of concentrations expected for both compounds. The method had no significant matrix effects, so it could be used to measure 1,3-PDO in different media without extraction or concentration steps.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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