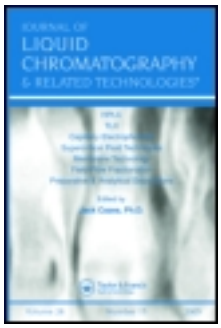

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/ljlc20

DEVELOPMENT OF AN HPLC METHOD FOR THE DETERMINATION OF GLYCEROL OXIDATION PRODUCTS

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Accepted author version posted online: 02 Jan 2013.

To cite this article: Juan Carlos Beltrán-Prieto , Jiří Pecha , Věra Kašpárková & Karel Kolomazník (2013): DEVELOPMENT OF AN HPLC METHOD FOR THE DETERMINATION OF GLYCEROL OXIDATION PRODUCTS, Journal of Liquid Chromatography & Related Technologies, DOI:10.1080/10826076.2012.725695

To link to this article: http://dx.doi.org/10.1080/10826076.2012.725695

Development of an HPLC method for the determination of glycerol oxidation products

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Abstract

An HPLC method for the separation of glycerol oxidation products, namely glyceraldehyde, dihydroxyacetone, mesoxalic, tartronic, glycolic and glyceric acids on an ion-exchange 8% crosslinked calcium sulfonated divinylbenzene-styrene resin column was developed and validated. The conditions reported include temperature (70 °C), flow rate (0.5 mL/min) and concentration of the mobile phase (3 mM H_2SO_4) using isocratic elution with ultraviolet and refractometric detectors. The effect of the mobile phase flow rate and concentration as well as column temperature on the resolution of peaks is described. Excellent correlation coefficient in the calibration model was observed for all analytes over the concentration range of 0.5 to 10 mg/mL. The method was also validated in terms of intra-day precision, sensitivity, accuracy, and detection and quantification limits. The method conditions were applied to the identification of products derived from the chemical oxidation of glycerol.

KEYWORDS: glycerol oxidation; HPLC method; ion exchange; validation;

1. INTRODUCTION

Glycerol (1,2,3-propanetriol) is an important side product of the triacylglycerol transesterification process. The search for alternative energy sources has increased its worldwide market as a result of the biodiesel production. Moreover, it is an important molecule from which several compounds can be formed through a variety of chemical reactions. The three hydroxyl groups, that the glycerol molecule contains, are susceptible to hydrogenation, (trans)esterification, dehydration, etherification, oxidation, pyrolysis, oligomerization, polymerization, and carboxylation. Among them, the oxidation of glycerol draws a special attention due to the practical valuable compounds that are formed as it is shown in Figure 1. Namely products of mild glycerol oxidation, such as dihydroxyacetone are of commercial value.[1] However, in practice, several glycerol oxidation products are formed simultaneously, due to the reactivity of primary and secondary hydroxyl groups.[2] As a result, the selectivity for a specific product is not easy to achieve and still remains as a challenge.[2] Therefore, it is important to have accurate and rapid methods for the analysis, identification and quantification of these compounds. Especially, an analytical method able to simultaneously determine all products of mild oxidation can significantly reduce and simplify the development of new catalysts and oxidation techniques. Such a method is also of value for the characterization of final products because it will in most cases contain also other substances formed during the glycerol oxidation due to the reason discussed above. Techniques such as Thin Layer Chromatography[3] and HPLC have been used for the identification, yield and quantitative determination of glycerol oxidation products. In particular, HPLC has been

broadly employed for this purpose as it provides short elution times and easy sample preparation with no need of derivatization.[4]

Many authors dealt with simultaneous determination of glycerol derivatives by means of HPLC. Analytical conditions of these published methods are listed in Table 1. As can be seen, cation exchange columns were mainly used, since they are able to facilitate separation of carbohydrates as well as simple organic acids (see Table 1).

Demirel et al.[5,7], used cation exchange column operated at 60 °C and 10 mM solution of sulfuric acid as a mobile phase to separate glycerol oxidation products. Virtually the same conditions were also used by Brandner.[6] The detailed insight into the analytical method and results published in[6,7] shows that under reported chromatographic conditions peaks of oxidation products, especially peaks of dihydroxyacetone (DHA) and glycerol, were overlapped. On the other hand, advantageous connection of ultraviolet (UV) and refractometric (RI) detectors in series allowed qualitative and quantitative determination of glycerol and DHA, since only DHA can be selectively identified on UV detector at 210 nm. Similar conditions with lower concentration of sulfuric acid (5 mM) were reported in.[10–13] Kwon and Koper[12] pointed out that namely peaks of glyceraldehyde and glyceric acid strongly overlap at lower temperatures (30 °C) and recommended to increase the column temperature up to 80 °C. The higher column temperature improved the difference between retention times of glyceraldehyde and glyceric acid from former value of 0.06 minutes to 0.93 minutes. However, the resolution between peaks of glyceraldehyde and glycolic acid was significantly reduced at higher temperature.

Analogous method is also described in papers[14–19,21–25] dealing with glycerol oxidation. Unfortunately, in some cases[20–26] the method conditions are not fully specified. In addition, to the best author's knowledge, these published methods were not rigorously optimized and validated (in terms of precision, accuracy, sensitivity, etc.) because the experiments were primarily focused on the process of glycerol oxidation and catalyst preparation. There is one exception, paper[21] of Chen et al., which focus on development of HPLC method and contains complete information about the analytical methodology and results of its validation. Chen et al.,[21] also reported problems with separation of glycerol and DHA using an HPX-87H ion exclusion column with H_2SO_4 as mobile phase in studied concentration range of 5 mM to 20 mM. The use of a mobile phase composed of deionized water-acetonitrile containing 0.5 mM H_2SO_4 (65:35) promoted the separation of glycerol and DHA with good resolution. However, this paper is limited to simultaneous determination of glycerol, DHA, 3-hydroxypropionaldehyde and 1,3-propanediol. Resolution improvement with similar mobile phase (i.e. deionized water-acetonitrile) was observed also in the paper;[19] nevertheless, the resolution limited to five glycerol oxidation products was presented and detailed validation data were not provided since the experimental work was not focused on analytical method development. To summarize the above information, the reliability of analytical methods for determination of glycerol oxidation products and derivatives is insufficient and can be further improved, especially in terms of peak identification, resolution and validation parameters.

The objective of this work was, hence, to optimize and validate an HPLC method for the simultaneous quantification of glycerol mild oxidation products, namely mesoxalic acid,

tartronic acid, glyceraldehyde, glyceric acid, glycerol, glycolic acid and dihydroxyacetone. The special emphasis was placed on the improvement of separation of overlapping peaks of glycerol, dihydroxyacetone, glycolic acid, glyceric acid and glyceraldehyde by optimizing the chromatographic conditions. Resolution between the consecutive peaks was calculated and validation parameters such as linearity, intra-day precision, accuracy, sensitivity, limit of detection and quantification, were determined. This is, to our best knowledge the first systematic study focused on optimization and validation the method for the simultaneous separation of a wide spectrum of glycerol oxidation products by means of HPLC.

2. EXPERIMENTAL

2.1. Instrument

Analyses were carried out using a modular Waters HPLC instrument with manual injection. The system comprises a Waters 600E pump, a vacuum degasser VD 040 (Watrex, Czech Republic), a refractive index detector (Waters 2414), and an ultraviolet detector UV200 (Watrex, Czech Republic). Data analysis and acquisition were performed with Clarity Chromatography Station.[27] A reversed-phase column Aminex HPX-87C, sulfonated divinylbenzene-styrene resin , 300 mm × 7.8 mm; (Bio-Rad) was employed for HPLC separation.

2.2. Chemicals And Reagents

Deionized water was used in all procedures (Millipore). Glycerol (Propane-1,2,3-triol), glyceraldehyde (2,3-Dihydroxypropanal), dihydroxyacetone (1,3-Dihydroxypropan-2-

one), tartronic acid (2-Hydroxypropanedioic acid), glycolic acid (2-Hydroxyethanoic acid), glyceric acid (2,3-Dihydroxypropanoic acid), and mesoxalic acid (Oxopropanedioic acid) standards were obtained from Sigma-Aldrich (Czech Republic). Sulfuric acid (H_2SO_4), chromium trioxide (CrO_3), sodium bicarbonate ($NaHCO_3$) and ethyl methyl ketone (Butan-2-one) were analytical grade chemicals purchased from Merck (Czech Republic).

2.3. Sample Preparation

Stock solutions of each standard were prepared. 100 mg of the respective compound were weighted, dissolved and diluted with deionized water to a final volume of 10 mL. Prior analyses, the samples were filtered through a nylon Millipore filter (0.22 □m). Standard solutions with concentrations ranging from 0.5 to 10 mg/mL were prepared for calibration. A 0.01 M H_2SO_4 stock solution was made by weighing 1.024 g of 95 % H_2SO_4 (0.54 mL), pouring it into a 1 L volumetric flask and diluting in deionized water. From this stock solution, mobile phases with different concentrations of sulfuric acid were prepared to be used for the method development.

For the purpose of oxidation of glycerol, Jones reagent was prepared by adding 5.75 mL of H_2SO_4 over 6.69 g of CrO_3 and the solution was diluted in deionized water (25 mL). Glycerol solution was prepared by weighing 1.7 g of the alcohol and diluting to 10 mL with ethyl methyl ether.

2.4. Chromatographic Method Development

The analysis of the glycerol oxidation products was performed using an ion exchange Aminex HPX-87C (300 mm × 7.8 mm) column in an isocratic mode with aqueous H_2SO_4 solution as mobile phase. Analytes were monitored by UV detection of carbonyl groups from carboxylic acids, ketones and aldehydes at 210 nm coupled in series with RI detector. In order to reveal the order of elution and the individual retention time of each of the standards, a first set of experimental conditions including mobile phase flow rate: 0.7 mL/min, temperature: 60 °C, and 0.01 M aqueous H_2SO_4 was used. The temperature of the refractometric detector remained constant at 30 °C. After the introductory experiment was conducted, a solution containing a mixture of the standards was analyzed and influence of the following variables on chromatographic separation was investigated; 1) temperature of 30, 60 and 70 °C 2) flow rate of 0.2, 0.5 and 0.7 ml/min and 3) concentrations of H_2SO_4 in mobile phase 1, 3, 5, and 10 mM. The final chromatographic conditions were set as follows: column temperature was increased to 70 °C, the injection volume used was 20 □L, flow rate of 0.5 ml/min, mobile phase with 3 mM H_2SO_4 and the temperature of the RI detector remained constant at 30 °C. Triplicates of all standards were analyzed.

2.5. Chromatographic Method Validation

Based on the results from method development given in section 3.1, the optimized method conditions were used for the method validation. Detection limit test was carried out by analyzing different concentrations of each compound. Dilutions were prepared sequentially from a solution that presented a signal to noise (S/N) ratio of at least 30 until the S/N ratio was approximately 3. The intra-day precision test was carried out by

performing seven replicate measurements at a specific concentration on the same day by the same analyst. For the HPLC calibration curves, six different concentrations of the standards (0.5, 1, 2, 5, 7, and 10 mg/mL) were prepared and evaluated by means of linear regression. Sensitivity of both detectors was measured by using a ±5 % variation in the response (mV·s) at six different concentrations (mg/mL). Accuracy was determined using the method of standard addition in terms of percent recovery. Three different fortified levels were prepared by adding solutions of specific concentration (2, 5 and 10 mg/mL) to a pre-analyzed, un-fortified sample. The percent recovery, *R* (%), was calculated using the equation (1), where *CB* represents the difference in concentration between the fortified and un-fortified samples, and *CA* stands for the concentration added in the fortified sample.

$$R\ (\%) = (CB / CA) \times 100 \qquad (1)$$

The standard score (Z-score) was calculated to know the closeness of the accordance between the concentrations measured in the sample and the standard reference solution as shown in equation (2), where σ represents the standard deviation of the population. Normally, Z-score values between -2 and 2 are considered to be acceptable.[28]

$$Z\text{-}score = (CA - CB) / \sigma \qquad (2)$$

Generally, during the development of a chromatographic method, a multivariate experimental design is recommended to optimize values for capacity factor (*k),*

selectivity (□, efficiency (*N*) and resolution (*Rs*) by changing variables such as mobile and stationary phase composition and temperature. Optionally it is possible to vary column conditions (flow rate, columns length or particle size).[29] As showed in equation (3), *Rs* is usually expressed as a function of *k,* □and *N*, therefore this value was used as the variable response for the optimization purpose. Calculation of *Rs* value for two adjacent peaks (represented as "*A*" and "*B*") was performed according to equation (4), where t_B and t_A corresponds to the retention time of both compounds, and *W* represents the bandwidths at half height of both peaks. For completely separated peaks, an *Rs* value higher than 1.5 is usually required.[30]

$$Rs = (1/4)\,(\alpha - 1)\,N^{1/2}\,\{k / (1 + k)\} \quad (3)$$

$$Rs = 1.18\,(t_B - t_A) / (W_{0.5,A} + W_{0.5,B}) \quad (4)$$

2.6. Method Application

The developed and validated method was applied to analyze the chemical oxidation products of glycerol obtained via reaction with Jones reagent. Briefly, 10 mL of a chromium trioxide solution in sulfuric acid were added dropwise to a 10 mL glycerol diluted solution (1.7 %) in an ice bath. The addition was slow and proceeded for approximately 20 min. After the entire chromium oxide solution was added, the reaction mixture was neutralized by the addition of a saturated aqueous solution of $NaHCO_3$ and 5 mL of ethyl methyl ketone were used for extraction. Finally, the extract containing oxidation products was filtered, diluted with water (1:10 ratio) and analyzed by means of HPLC.

3. RESULTS AND DISCUSSION

3.1. Method Development

The primary aim of this work was to improve separation of glycerol mild oxidation products, namely mesoxalic acid, tartronic acid, glyceraldehyde, glyceric acid, glycerol, glycolic acid and dihydroxyacetone. As it was written above, the simultaneous analysis of these products is complicated by poor resolution resulting in peak overlapping. In order to suppress this phenomenon, the effect of column temperature, flow rate and concentration of aqueous H_2SO_4 (used as mobile phase) was examined; it was observed that the separation of glycerol oxidation products on a sulfonated divinylbenzene-styrene resin column is dependent on all these factors.

Analytical conditions which were examined during method optimization, with the corresponding *Rs* values are listed in Table 2. At first, mobile phase comprising of 0.01 M H_2SO_4, temperature of 60 °C and flow rate of 0.7 mL/min were used. These preliminary tests showed that namely peaks of glyceraldehyde – glyceric acid – glycerol (first group) and peaks of glycolic acid – DHA (second group) were overlapped as it was indicated in the literature. The effort was therefore focused on the improvement of resolution between substances in these two groups. Due to the fact that the RI detector allows the identification of all the compounds, the *Rs* values reported in Table 2 are for this type of detector. The experiments showed that resolution between glyceraldehyde and glyceric acid increased with the raise in flow rate and concentration of sulfuric acid

in mobile phase as it is presented also in Supplemental Figure 1. A maximum resolution of 1.26 between these two substances was achieved at a flow rate of 0.7 mL/min and with 10 mM H_2SO_4 (pH=1.7). On the contrary, decreasing the concentration of H_2SO_4 below 5 mM ($2 < pH < 2.7$) improved the separation between glyceric acid and glycerol, allowing the qualitative determination of these compounds. However, additional decrease of concentration up to 1 mM ($pH > 2.7$), did not further improved separation as illustrated in Figure 2. In fact, the glyceraldehyde peak overlapped with glyceric acid and glycerol peak started to overlap with glycolic acid at these conditions. A similar effect was observed for peaks of mesoxalic and tartronic acids, both showing reduced resolution at lower concentrations of sulfuric acid. Figure 2 clearly documents the influence of sulfuric acid concentration on separation between all compounds. The use of 3 mM H_2SO_4 as mobile phase with a flow rate of 0.5 mL/min allowed a resolution of 0.6 between glycerol and glyceric acid. However, glyceric acid can be identified with good resolution and without overlapping by UV detector, since glycerol does not show absorption at the wavelength applied. Moreover, the decrease of H_2SO_4 concentration positively influences the *Rs* value between glycolic acid and dihydroxyacetone (as illustrated also in Supplemental Figure 2). In summary, the choice of the sulfuric acid concentration presents a compromise between resolution of glyceraldehyde and glyceric acid on the one hand and glyceric acid and glycerol on the other hand. The best resolution between these three compounds was achieved with 3 mM H_2SO_4 at a flow rate of 0.5 mL/min, showing *Rs* values of 3.13 (mesoxalic and tartronic acid), 0.73 (glyceraldehyde and glyceric acid), 0.61 (glyceric acid and glycerol) and 1.27 (glycolic acid and dihydroxyacetone).

The separation was also significantly affected by temperature. At 30 °C, the peaks were highly asymmetric with a notable fronting observed. However, this situation was overcome by increasing the column temperature to 70 °C. A similar observation was done by Moore and Stein[31] during the separation of amino acids using a Dowex-50, 0.9 × 100 cm column, in the sodium form with different temperature conditions and mobile phase with buffers that progressively increased the pH. The temperature of 70 °C was used based on the recommendations of the column manufacturer, as usually a maximum operating temperature of 80-85 °C is proposed to avoid damage of the column.

The best overall results for the separation of seven different glycerol oxidation products were achieved using a concentration of 3 mM H_2SO_4, 70 °C, and flow of 0.5 mL/min. At these conditions, it is possible to clearly distinguish all compounds of interest. Though, the peaks of glyceraldehyde, glyceric acid and glycerol are still overlapped, these conditions allow for better separation of glycerol oxidation products in comparison with published results. Especially glycerol and DHA are clearly separated and the resolution values between other compounds (e.g. glyceraldehyde and glyceric acid) were improved. Hence, these conditions were chosen as acceptable and reliable for the simultaneous determination of oxidation products and the method was further validated in order to examine its accuracy for the purposes of quantification.

3.1.2. Validation Parameters

Quantification of glycerol oxidation products was performed by means of calibration curves based on the UV and RI spectrophotometric response of known amounts of the

standards in aqueous solutions. Table 3 presents the analyses in a concentration range of 0.5-10 mg/mL. A flow rate of 0.5 mL/min was used with 3 mM H_2SO_4 as mobile phase at 70 °C. Linearity was determined by means of the calculation of the linear least square regression. All calibration curves showed a good linear correlation ($r^2 > 0.999$) within the entire concentration range used.

The relative standard deviation (RSD) is presented as a percentage in Table 4. The range is from 2.3 to 4.2 % for the UV detector and from 1.75 to 6.39 % for the RI detector which indicates satisfactory values for precision of the instrument. Similarly, the detector sensitivity test performed at six different concentrations showed acceptable RSD values, as presented in Table 5. In order to determine the detection limit, the respective concentration was taken into consideration when the S/N ratio in triplicate exceeded the value of three. Under this condition, the concentrations of each compound that could be detected by the instrument are reported in Table 5. The limit of quantification (LOQ) is included and was calculated according to.[32] Linearity plot presented in Figure 3 illustrates the dependence between sensitivity and concentration showing the ranges of constant response for glyceric acid within a 5 % level of deviation. The method showed reliable quantification over the range of 1 to 10 mg/mL for all the compounds and using 8 % deviation the value of 0.5mg/ml is also inside the linear response for all the standards. Values outside the linear range of the detector sensitivity can be considered as the limit of quantification, however according to Ribani et al.,[32] the value obtained by the signal to noise ratio is generally lower than the one obtained by the sensitivity test.

3.2. Method Application

In order to verify the method performance on a real sample, the products of chemical glycerol oxidation were analyzed. The chromatogram of the sample from Jones oxidation of glycerol is presented in Figure 4. It was seen that the real oxidation products were clearly identified using the developed and validated HPLC method. The concentrations of the products were: glycerol (90 ±0.04 mg/mL), glyceraldehyde (4.14 ±0.03 mg/mL), glyceric acid (5.85 ±0.029 mg/mL), and dihydroxyacetone (1.54 ±0.036 mg/mL). Accuracy was determined using the sample obtained from the oxidation of glycerol as un-fortified solution. Table 6 presents the percent recoveries and Z-score at the respective fortified level for each compound determined in triplicate. In all cases, it was found a recovery from 96.5 to 103.3 % (both detectors) for the studied levels and a Z-score within the acceptance limits of -2 to 2. The combination of partial separation and selective response of the RI and UV detectors allowed the reliable quantification of the analytes. This demonstrates the method suitability in the identification and also quantification of glycerol oxidation products by HPLC.

4. CONCLUDING REMARKS

There are only a few HPLC methods published that discuss the identification and separation of glycerol oxidation compounds. Moreover, these methods do not clearly state a methodology for the optimization of the chromatographic conditions and the results of their rigorous validation were not reported. In this work, a simple method capable of simultaneous determination of seven different glycerol oxidation products was proposed. Though this method is based on previously published procedures, the

separation of oxidation compounds was noticeably improved and chromatographic conditions that allowed reproducible elution of individual peaks and acceptable resolution between analytes with closer retention time were proposed. The proposed method implies the use of a sulfonated divinylbenzene-styrene resin column in the calcium ionic form (Aminex HPX-87C), which was effective for the analysis with a flow rate of 0.5 mL/min, 3 mM of H_2SO_4 as mobile phase, and a temperature of 70 °C.

Since a simultaneous separation of such a number of glycerol derivatives with similar structure is a challenging task, the peak overlapping was not fully eliminated with the optimized method; however, it was noticeably reduced in comparison to currently known methods. Due to the reason that the simple simultaneous determination is of significant practical value, this method was further validated and its accuracy was successfully verified with the real sample of oxidation products. Briefly, the results of intra-day precision, detection and quantification limit, linearity, accuracy and sensitivity were reported. It was therefore proved that the method is reliable enough and thus useful e.g. in the area of new glycerol oxidation processes development.

ACKNOWLEDGEMENTS

The project was financially supported by the European Regional Development Fund (ERDF) under the Project CEBIA-Tech No. CZ.1.05/2.1.00/03.0089, with support of Operational Program Research and Development for Innovations co-funded by the ERDF and national budget of Czech Republic, within the framework of project Centre of Polymer Systems (reg. number: CZ.1.05/2.1.00/03.0111) and Tomas Bata University

Internal Grant (IGA/FAI/2012/029). One author (B-P.) is also grateful for the doctoral scholarship provided by Tomas Bata University in Zlín and the National Council of Science and Technology (CONACYT) in Mexico.

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Table 1. Method conditions reported in literature for the identification of glycerol oxidation products by HPLC.

Column	Mobile phase	Column temperature (°C)	Reference
Aminex HPX-87H	10 mM H_2SO_4	60	[5,6]
Aminex HPX 87C	10 mM H_2SO_4	60	[7]
Aminex HPX-87H	5 mM H_2SO_4	60	[8,9]
Aminex HPX-87H	5 mM H_2SO_4	55	[10]
Aminex HPX 87H	5 mM H_2SO_4	45	[11]
Aminex HPX-87H	5 mM H_2SO_4	30	[12]
Alltech IOA 1000	5 mM H_2SO_4	25	[13]
Alltech QA-1000	4 mM H_2SO_4	70	[14]
Aminex HPX-87H	4 mM H_2SO_4	30	[15]
ICPak Ion Exclusion	0.4 mM H_2SO_4	70	[16]
Hitachi GL-C610-S	Water	60	[17,18]
Zorbax SAX	H_3PO_4 (0.1 % w/w) in H_2O-acetonitrile (1/2 v/v)	25	[19]
Alltech OA-1000	10 mM H_2SO_4/10 mM H_3PO_4	Not reported	[20]
Aminex HPX-87H	65:35 H_2O–acetonitrile containing 0.5 mM H_2SO_4		[21]
Aminex HPX-87H	0.01 M H_2SO_4		[22]
Rezex ROA	0.01 N H_2SO_4		[23]

Alltech OA-1000	Not reported		[24]
Zorbax SAX			[25]
Sarasep Car-H			[26]

Table 2. Summary of the experiments realized for the identification of the standards analyzed with the respective resolution achieved using refractive index detector.

Temperature (°C)	H_2SO_4 (mM) in mobile phase	pH	Flow rate (mL/min)	Resolution[a)]			
				Mesoxalic-Tartronic acid	Glyceraldehyde-Glyceric acid	Glyceric acid-Glycerol	Glycolic acid-Dihydroxyacetone
70	10	1.7	0.7	3.5	1.2	0	0.74
70	5	2	0.7	3.4	1.59	0	1.06
70	3	2.2	0.7	3.1	0.96	0.51	1.22
70	1	2.7	0.7	2.68	0.85	0.5	1.32
70	10	1.7	0.5	3.172	1.25	0	0.88
70	5	2	0.5	3.45	0.87	0	1.11
70	3	2.2	0.5	3.11	0.73	0.61	1.27
70	2	2.4	0.5	3	0.85	0.56	1.35
70	1	2.7	0.5	2.67	0.51	0.58	1.44
70	10	1.7	0.2	3.08	0.59	0	1.04
70	5	2	0.2	3.17	0.54	0	1.23
70	3	2.2	0.2	2.83	0.19	0.8	1.35
70	1	2.7	0.2	2.26	0	1.15	1.49
60	10	0.01	0.7	2.48	0.99	0	0.67
60	10	0.01	0.8	2.64	0.93	0	0.66

a) Resolution was calculated according to the expression (4)

Table 3. Standard curves for glycerol oxidation products.

Standard	Retention time (min)	Equation[a] ($y=ax$)			
		UV detector		RI detector	
		a (mV·s·mL/mg)	r^2	*a* (mV·s·mL/mg)	r^2
Mesoxalic acid	9.2	3653.8	0.999	1054.0	0.999
Tartronic acid	10.8	5111.2	0.999	1235.9	0.999
Glyceraldehyde	17.2	731.7	0.999	1483.1	0.999
Glyceric acid	18.5	1213.6	0.999	885.4	0.999
Glycerol	19.3			1244.1	0.999
Glycolic acid	21.4	1380.0	0.999	981.7	0.999
Dihydroxyacetone	23.0	1572.8	0.999	1224.6	0.999

[a]Range 0-10 mg/mL

Table 4. Intra-day precision test of the HPLC method for the determination of glycerol oxidation products expressed as a peak area

Standard	UV detector			RI detector		
	Mean peak area (N = 7) (mV·s)	Standard deviation	RSD (%)	Mean peak area (N = 7) (mV·s)	Standard deviation	RSD (%)
Mesoxalic acid	588.1	14.1	2.4	200	5.5	2.7
Tartronic acid	920.4	21.2	2.3	226.5	6.8	3
Glyceraldehyde	102.8	4.3	4.2	246	15.7	6.4
Glyceric acid	203.2	5.7	2.8	384.7	6.8	1.7
Glycerol	n.a	n.a	n.a	206.5	3.14	1.4
Glycolic acid	177.2	7	3.9	129.4	5.1	3.9
Dihydroxyacetone	192.5	6.5	3.3	152.2	6.1	4

n.a. not applicable

Table 5. Determination of sensitivity using UV and RI detectors and determination of LOD and LOQ values for each compound

Compound	UV detector		RI detector		LOD	LOQ
	Mean Sensitivity (mV·s·mL/mg)	RSD (%)	Mean Sensitivity (mV·s·mL/mg)	RSD (%)	(mg/mL)	(mg/mL)
Mesoxalic acid	3865.2	5.3	1075.1	3.1	0.013	0.043
Tartronic acid	5408.3	5.4	1249.5	2.4	0.014	0.046
Glyceraldehyde	703.0	4.3	1494.8	1.5	0.017	0.056
Glyceric acid	1223.3	2.1	901.7	3.9	0.010	0.033
Glycerol	n.a	n.a	1266.2	3.0	0.015	0.049
Glycolic acid	1387.9	1.7	995.6	3.2	0.010	0.033
Dihydroxyacetone	1598.0	2.0	1238.4	1.7	0.014	0.046

n.a. not applicable

Table 6. Accuracy test expressed as Percent recovery

		RI detector			UV detector		
Compound	Fortified level (mg/mL)	Detected Level (mg/mL)	Recovery (%)	Z-score	Detected Level (mg/mL)	Recovery (%)	Z-score
Glycerol	2	1.95	97.50	-1.77	n.a.	n.a.	n.a.
	5	4.94	98.80	-1.69	n.a.	n.a.	n.a.
	10	9.90	98.97	-1.80	n.a.	n.a.	n.a.
Glyceraldehyde	2	2.05	102.33	0.37	2.01	100.67	0.18
	5	4.96	99.27	-0.75	4.95	99.07	-0.95
	10	10.04	100.37	0.75	10.05	100.47	0.71
Glyceric acid	2	1.94	96.83	-1.72	1.93	96.50	-0.64
	5	4.94	98.73	-1.92	5.05	101.00	0.59
	10	9.83	98.33	-1.76	10.06	100.63	0.93
DHA	2	2.02	101.17	0.26	2.03	101.67	1.27
	5	4.97	99.33	-0.35	5.07	101.40	1.08
	10	9.93	99.33	-0.96	10.33	103.30	0.78

n.a. not applicable

Figure 1. Glycerol oxidation pathway.

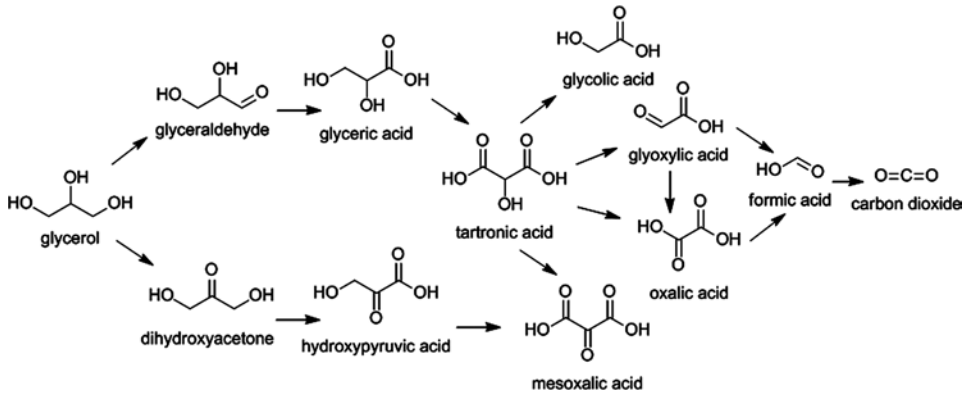

Figure 2. Chromatograms of a mixture of standards using concentrations of sulphuric acid from 1 mM to 5 mM. a) 1 mM, b) 2 mM, c) 3 mM, d) 5 mM, e)10 mM at 70 °C and flow rate of 0.5 mL/min. Description of peaks is as follows: 1) Mesoxalic acid, 2) Tartronic acid, 3) Glyceraldehyde, 4) Glyceric acid, 5) Glycerol, 6) Glycolic acid, 7) Dihydroxyacetone

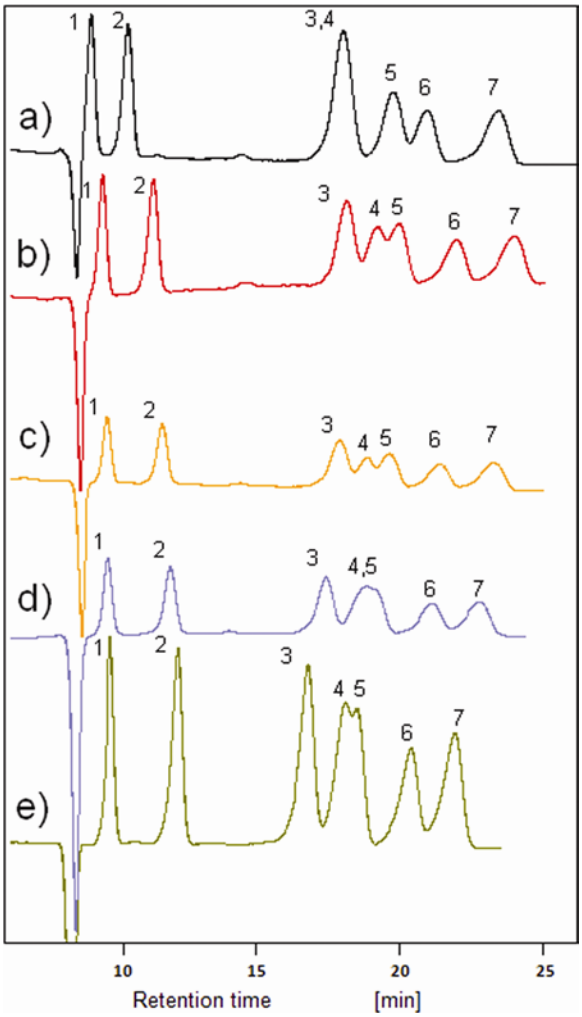

Figure 3. Sensitivity test for quantification of glyceric acid in RI detector.

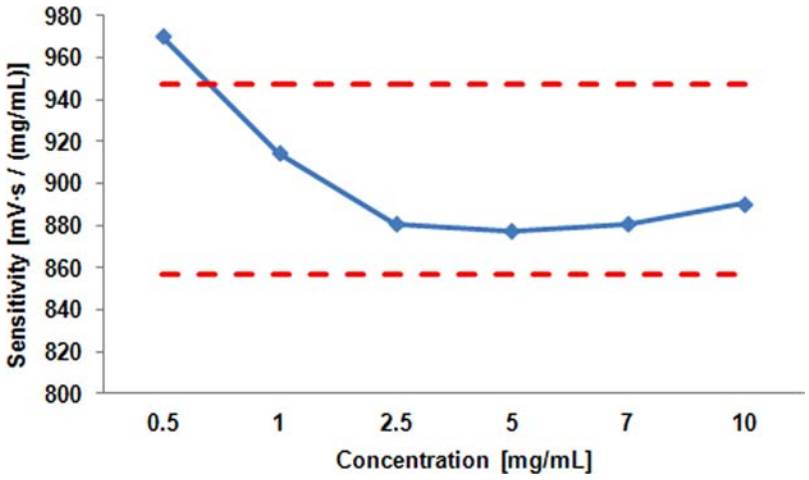

Figure 4. Chromatogram of the products obtained by the chemical oxidation of glycerol using Jones reagent. Flow rate: 0.5 mL/min, mobile phase: 3 mM H_2SO_4, temperature: 70 °C. Label of peaks: 1) Glyceraldehyde (17.1 min), 2) Glyceric acid (18.4 min), (3) Glycerol (20.1 min), and 4) Dihydroxyacetone (23.2 min). Peak 5) (27.1 min) corresponds to ethyl methyl ether used as solvent.

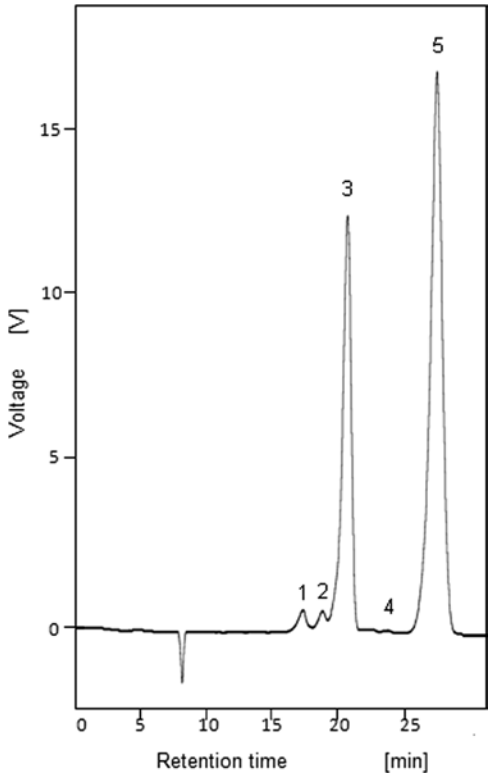

Figure 5. Influence of the concentration of the mobile phase [mM H_2SO_4] and flow rate [mL/min] in the resolution of glyceraldehyde and glyceric acid.

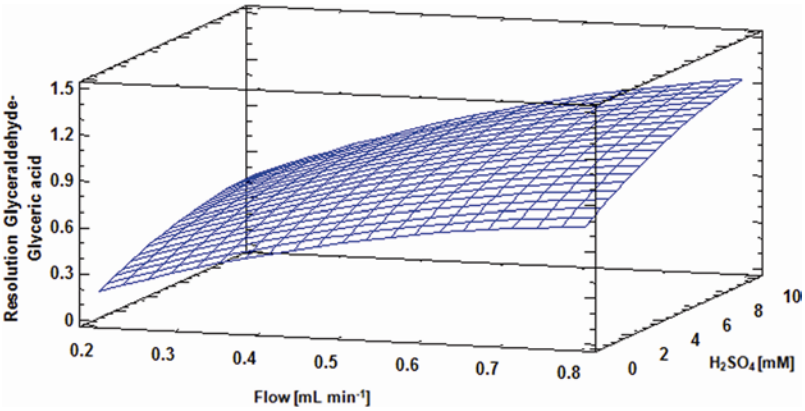

Figure 6. Influence of the concentration of the mobile phase [mM H_2SO_4] and flow rate [mL/min] in glycolic acid and dihydroxyacetone resolution.

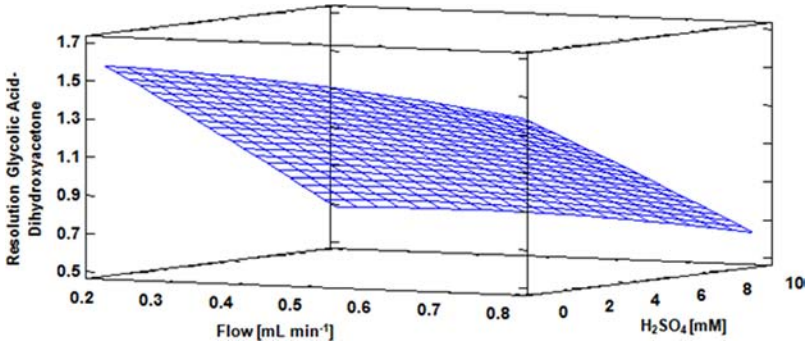

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Figure 7. Influence of the concentration of the mobile phase [mM H_2SO_4] and flow rate [mL/min] in mesoxalic and tartronic acid resolution.