Determination of glycerol derivatives by High-performance liquid chromatography

JUAN CARLOS BELTRÁN PRIETO\textsuperscript{a}, JIŘÍ PECHA\textsuperscript{a}, VĚRA KAŠPÁRKOVÁ\textsuperscript{b,c}, KAREL KOLOMAZNÍK\textsuperscript{a}

\textsuperscript{a}Department of Automation and Control Engineering, \textsuperscript{b} Centre of Polymer Systems, University Institute, \textsuperscript{c}Department of Fat, Surfactant and Cosmetics Technology Tomas Bata University in Zlín

\textsuperscript{a}Nám. T. G. Masaryka, 5555, \textsuperscript{b}Nad Ovčírnou 3685, \textsuperscript{c}nám. T. G. Masaryka 275, 760 01 Zlín CZECH REPUBLIC

prieto@fai.utb.cz, pecha@fai.utb.cz, vkasparkova@ft.utb.cz, kolomaznik@fai.utb.cz

Abstract: A chromatographic method for the identification of glycerol derivatives, particularly 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 experiments included a systematic study of the effect of column temperature (60, 70 °C); flow rate (0.2, 0.5, 0.7, and 0.8 mL min\textsuperscript{-1}) and mobile phase conditions of aqueous H\textsubscript{2}SO\textsubscript{4} (1, 3, 5, and 10 mM) on the separation and quantification of the glycerol oxidation products using isocratic elution with Ultraviolet and Refractometric detectors. Best results were achieved using a flow rate of 0.5 mL min\textsuperscript{-1}, with 3 mM H\textsubscript{2}SO\textsubscript{4} as mobile phase at 70 °C. The method was validated in terms of intra-day precision, sensitivity, accuracy, and detection and quantification limits. The separation mechanism was studied and the method conditions were applied in the identification of products derived from the chemical oxidation of glycerol.

Key-Words: - HPLC analysis, glycerol derivatives, chromatographic method, validation, UV detector, RI detector, ionic exchange

1 Introduction
Glycerol (1,2,3-propanetriol) it is an important molecule that has increased its worldwide market presence as a result of the biodiesel production in the search for alternative energy sources. In addition, 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. However, in practice several glycerol derivatives are formed simultaneously, due to the reactivity of the primary and secondary hydroxyl groups [1]. As a result, the selectivity for a specific product is not easy to achieve and still remains as a challenge.

For this reason, 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 in most cases it will also contains other substances formed during the reaction. Many authors have dealt with simultaneous determination of glycerol derivatives by means of HPLC. However, under reported chromatographic conditions [2, 3], peaks of oxidation products such as dihydroxyacetone (DHA) and glycerol, were overlapped. In other cases [4], peaks of glyceraldehyde and glyceric acid strongly overlapped at lower temperatures (30 °C).

Unfortunately, in the majority of the cases, the method conditions are not fully specified and have not been rigorously optimized and validated. Therefore, 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 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. Special emphasis was placed on improving the separation of overlapping peaks of glycerol, dihydroxyacetone, glycolic acid, glycric 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 Problem Formulation

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 [29].

2.1 Chromatographic method development

The analysis of glycerol oxidation products was performed using an ion exchange Aminex HPX-87C (300 mm x 7,8 mm) column in isocratic mode with aqueous H$_2$SO$_4$ solution as mobile phase and monitored at 210 nm by UV detection of carbonyl compounds from carboxylic acids, ketones and aldehydes. In order to reveal the order of elution and the individual retention time of each standards, a first set of experimental conditions including flow: 0.7 ml min$^{-1}$, temperature: 60 °C, and 0.01 M H$_2$SO$_4$ as mobile phase 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 at 30 and 60 °C under the same conditions. In order to optimize the mobile phase composition for the HPLC analysis, different flow rates (0.2, 0.5, 0.7 and 0.8 mL min$^{-1}$) and mobile phase conditions of aqueous H$_2$SO$_4$ (1, 3, 5, and 10 mM) were tested and the column temperature was increased to 70 °C. The injection volume used was 20 µL and the temperature of the RI detector remained constant at 30 °C. Triplicates of all standards were analyzed.

2.2 Chromatographic validation

Detection limit test was carried out by analyzing different concentrations of glycric acid, the compound that showed the less response in RI detector. 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 performed to know the variability in measurements between experiments in terms of the peak-area ratios at a specific concentration on the same day. For the HPLC Calibration curves, six different concentrations of the standards (0.5, 1, 2, 5, 7, and 10 mg ml$^{-1}$) were prepared and the suitability was analyzed by means of linear regression. Sensitivity of both detectors was determined by using the variability in the response (mV·s) at six different concentrations (mg mL$^{-1}$). Accuracy was determined by using the method of standard addition and in terms of percent recovery. Three different fortified levels were prepared by adding solutions of specific concentration (2, 5 and 10 mg ml$^{-1}$) to a pre-analyzed sample (un-fortified solution).

2.3 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 product was filtered, diluted with water (1:10 ratio) and analyzed by means of HPLC.

3 Problem Solution

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. The simultaneous analysis of these products is complicated by poor resolution causing peak overlapping. In order to suppress this phenomenon, the effect of column temperature, flow rate and concentration of aqueous H$_2$SO$_4$ (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. First of all, a mobile phase comprising 0.01 M H$_2$SO$_4$, a temperature of 60 °C and a 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. 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 Fig.1.

Fig.1, Influence of the concentration of the mobile phase [mM H$_2$SO$_4$] and flow rate [mL min$^{-1}$] in the resolution of Glyceraldehyde and Glyceric acid.

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$_2$SO$_4$ (pH=1.7). On the contrary, decreasing the concentration of H$_2$SO$_4$ 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 the separation as illustrated in Fig.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. Fig.2 clearly documents the influence of sulfuric acid concentration on separation between all compounds.

Fig.2, Chromatograms of a mixture of standards using concentrations of H$_2$SO$_4$ between 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.

The use of 3 mM H$_2$SO$_4$ 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 could be identified with good resolution and without overlapping by UV detector, since glycerol does not show absorption at the wavelength applied. Moreover, as illustrated in Fig.3, the decrease of H$_2$SO$_4$ concentration positively influences the $Rs$ value between glycolic acid and dihydroxyacetone 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$_2$SO$_4$ 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).

Fig.3, Influence of the concentration of the mobile phase [mM H_2SO_4] and flow rate [ml min^{-1}] in Glycolic acid and Dihydroxyacetone resolution.

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. 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^{-1}. 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.

3.2 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. 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.

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, a concentration of 0.01 mg/mL could still be detected by the instrument. The limit of quantification (LOQ) calculated was 0.033 mg/mL at S/N ratio=10. The range of relative standard deviation (RSD) 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. Linearity plot presented in Fig.4 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.

Fig.4, Sensitivity test for quantification of glyceric acid in RI detector.

3.3 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 Fig.5. It was seen that the real oxidation products were clearly identified using the developed and validated HPLC method. The concentrations of the products were: glyceraldehyde (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. 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.

3.4 Analysis of the separation mechanism

When the eluent enters to the column, the equilibrium of the ionic strength in the packing material starts to take place to allow the binding of the analytes. The presence of aqueous media in the mobile phase helps to swell the stationary phase and make it permeable. Once the molecules interact with the resin, different degrees of adsorption occur by reversible bindings with the support. The separation is achieved due to the individual properties of each molecule to interact with the cation exchanger due to differences in their charges, charge densities, distribution on their surfaces and nature of its functional group. In addition, the affinity of the support for ionic and non ionic parts of the molecule is an important factor that also determines the degree of separation. The degree of ionization and the repulsive force are proportional to the pH of the medium and therefore to the pKa of the analyte. Organic acids are separated through partitioning and ion exclusion mechanism. In this research, an elution order according to the increase of pKa was observed. The pH of the eluent and temperature can influence the pKa value. Decreasing the concentration of acid media in the mobile phase causes the solutes to reduce the degree of protonation and consequently they become less positively charged.

4 Conclusion

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, 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.

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