Analysis of Glyoxal and Related Substances by Means of High-Performance Liquid Chromatography with Refractive Index Detection

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A simple and rapid method is described for the analysis of glyoxal and related substances by high-performance liquid chromatography with a refractive index detector. The following chromatographic conditions were adopted: Aminex HPX-87H column, mobile phase consisting of 0.01N H2SO4, flow rate of 0.8 mL/min and temperature of 65°C. The application of the analytical technique developed in this study demonstrated that the aqueous reaction mixture produced by the oxidation of acetaldehyde with HNO3 was composed of glyoxal, acetaldehyde, acetic acid, formic acid, glyoxylic acid, oxalic acid, butanedione and glycolic acid. The method was validated by evaluating analytical parameters such as linearity, limits of detection and quantification, precision, recovery and robustness. The proposed methodology was successfully applied to the production of glyoxal.

Introduction

Glyoxal (Chemical Abstracts Service number: 107-22-2; International Union of Pure and Applied Chemistry name: oxaldehyde) is an organic compound with the formula OCHO. Anhydrous glyoxal has a melting point of approximately 15°C. However, it is typically supplied as an aqueous solution (usually containing 30–50% glyoxal) in which hydrated oligomers are present (1). Glyoxal is used as a chemical intermediate in the production of pharmaceuticals and dyestuffs, as a non-formaldehyde cross-linking agent in the production of a range of different polymers, as a biocide and as a disinfecting agent. A novel process for producing high-quality glyoxal by the liquid phase oxidation of acetaldehyde with dilute nitric acid was developed in the authors’ laboratory. A theoretical analysis of the reaction system showed that some acetic acid, glyoxylic acid, oxalic acid and glycolic acid would be produced as by-products during the oxidation; that is, these substances coexist in the reaction mixture. Because the system is complex and the structures of some of these substances are similar, qualitative and quantitative analysis is a substantial problem.

Many existing methods and techniques are not satisfactory for the analysis of glyoxal. Because glyoxal molecules have weak or no absorption in the ultraviolet (UV) and visible light regions, they cannot be directly detected by spectrophotometry. Therefore, the measurement is always conducted after derivatization and the use of high-performance liquid chromatography (HPLC) or another instrument. However, this leads to low precision for the analysis of glyoxal. Several papers have reported the instrumental analysis of glyoxal. Edelkraut and Brockmann (2) detected and quantified glyoxal in water samples by using the typical 2,4-DNPH derivatization followed by HPLC with diode array detection at 360 nm. They reported a detection limit of 295 ng/L. Glaze et al. (3) employed aqueous-phase PFBHA derivatization, yielding the corresponding pentfluoro-benzyl oxime, followed by hexane extraction and detection by gas chromatography (GC)–electron capture detection (ECD) or GC–mass spectrometry (MS). A minimum detection limit of 5.1 μg/L was obtained using GC–ECD, whereas GC–MS detection resulted in a minimum detection limit of 7.7 μg/L. As a viable alternative, derivatization using o-phenylenediamine to measure the corresponding quinoxaline before HPLC–UV detection has been described (4). Schramm and Rinderer (5) investigated the determination of cotton-bound glyoxal via an internal Cannizzaro reaction by means of HPLC. Chen et al. (6) developed a new method that combines ion chromatography with a conductivity detector to separate and analyze mixtures of glyoxal, glycolic acid, oxalic acid and glyoxylic acid. Both of these methods analyzed glyoxal by converting it to glycolic acid in the presence of a strong base. Due to the long reaction time for glyoxal derivatization, the analysis process was time-consuming and always led to large errors. Furthermore, the methods mentioned previously present other disadvantages, such as the requirement of many chemical reagents and low sensitivity.

Most of the instrumental analysis methods have been used for the qualitative and quantitative determination of glyoxal only; they cannot be used to analyze the other by-products that are produced during the reaction. The chemical analysis method has been the primary method used in the commercial production of glyoxal. Total aldehydes have been estimated by using sodium periodate. Nitric and organic acids have been estimated by titrating against alkali by using different indicators (7). However, the chemical analysis method cannot offer the possibility of qualitative evaluation of all components. The concentrations of acetic, glyoxylic, formic, glycolic and oxalic acids cannot be obtained; therefore, it is not possible to evaluate the degree of oxidation of acetaldehyde needed to improve the process. Furthermore, the chemical analysis method has the following inherent disadvan-
tages: (i) sensitivity of operating steps to a variety of factors, (ii) time-consuming detection, and (iii) interference. It is important to develop a fast and reliable analytical method that can simultaneously determine glyoxal and other reaction by-products.

To date, no analytical method exists that allows the qualitative and quantitative determination of all components in the aqueous reaction mixture produced by the oxidation of acetaldehyde with nitric acid. Beyond simple technical details, little is known about the reaction itself. Therefore, the present study is focused on the quantitative analysis of the glyoxal reaction solution by HPLC using a differential refractive index detector (RID). This method is important because the complete analysis of the oxidation mixture is required to evaluate reaction selectivity, kinetic analysis and choice of appropriate process parameters for the optimal product yield. It will also allow improved understanding of the oxidation reaction mechanism.

**Experimental**

**Chemicals**

All chemicals were of analytical or HPLC grade and all solutions were prepared from deionized water. Glyoxal (40%, w/w), oxalic acid (99.5%, w/w), formic acid (97%, w/w), glyoxylic acid monohydrate (97%, w/w) and glycolic acid (98%, w/w) were purchased from Merck (Darmstadt, Germany). Acetic acid (99.5%, w/w), acetaldehyde (98.5%, w/w) and H₂SO₄ (98%, w/w) were obtained from Ke Wei Co. (Tianjin, China).

**Instrumentation**

The HPLC analyses were performed on an LC-20A (Shimadzu, Kyoto, Japan) instrument equipped with a degasser, LC-20AB pump, autosampler and Aminex HPX-87H column, which had dimensions of 300 x 7.5 mm and was packed with strong cationic exchange resin (Bio-Rad Labs, Richmond, CA). The column was thermostated in a Shimadzu CTO-20A oven compartment. The system included a 20 μL loop and an RID-10A (Shimadzu) refractive index detector. Data acquisition was conducted with LC Solution 1.22 sp1 chromatography software (Shimadzu).

**Selection of detector and chromatography column**

The sample contained both organic and inorganic acids, including acetic acid, glyoxylic acid, oxalic acid and nitric acid. Ion-exclusion chromatography was recognized as a useful technique for the separation of organic and inorganic weak acids (8,9). Glyoxylic acid and glyoxal are thermo-sensitive materials that have weak or no absorption in the UV or visible light regions. Furthermore, the inorganic anions in the solution interfered with conductivity detection. By contrast, RID detectors have been widely used as general purpose detectors. They are especially suitable for routinely analyzing weak or non UV-absorbing substances. Therefore, an RID detector was selected for the research presented in this article.

The selection of a chromatographic column was another important aspect in developing an effective HPLC method. The Aminex HPLC column packed with polystyrene divinyl benzene resin has good properties, such as pH stability and high pressure resistance, column efficiency and selectivity. Resin-based HPLC columns can separate materials based on the mechanisms of ion exclusion, ion exchange, ligand exchange, size exclusion and reversed-phase and normal-phase partitioning. These multiple interaction modes offer a unique ability to separate compounds. The Aminex column is commonly used in the industry to analyze carbohydrates, organic acids, organic bases and other small organic molecules in standard products such as peptides and nucleic acids (10–12). Many different Aminex columns are dedicated to different classes of compounds. In this work, an Aminex HPX-87H column was selected to conduct the investigation. In this case, two primary kinds of separation mechanisms operated during the separation process: ion exclusion and reversed-phase partitioning. The two types of solutes should be reported as (i) polar ionizable compounds whose ionization degrees depend on the pH of the mobile phase, (ii) neutral molecules that are more or less polar. Solutes that are partially ionized had an intermediate behavior. Ions bearing a charge of the same sign as the functional groups of the stationary phase (sulfonate groups) are excluded by electrostatic repulsion, whereas neutral molecules are not. Therefore, a key to the retention mechanism for compounds that are ionizable is their ionization degree (or effective charge), and their retention time results from the action between repulsion and partition, because at a given pH, the ionized fraction cannot be separated from the molecular fraction. This is why the retention characteristics and the resolution depend on the sulfuric acid concentration. Therefore, organic acids are eluted according to their constant acidity values. For neutral molecules, only their hydrophobicity plays a role, which shows that reversed-phase partitioning is involved in the separation. In this case, the more hydrophobic molecules eluted later than the less hydrophobic molecules, as manifested by the increasing retention time. At the same time, nitrate ions were totally excluded and eluted at the void volume. Experimental results showed that the Aminex HPX-87H column could effectively separate the sample components.

**Selection of diluting solvent**

Generally, the negative peak caused by water has little effect on analysis; in this system, however, water may affect oxalic acid analysis. When using water as a diluent, the large negative peak will impede the oxalic acid analysis because they have similar retention times. During this study, all standard and sample solutions were diluted in the mobile phase (dilute sulfuric acid) to eliminate the disturbance caused by the negative peak of water.

**Determination of chromatographic conditions**

To establish the operating conditions that provide the maximum component peak resolution and minimum analysis time (i.e., elution time), the effects of parameters that influence resolution and analysis time must be investigated. With Aminex columns, several chromatographic variables have an impact on resolution and retention times and help to control the speed of analysis. These variables included eluent composition, eluent pH, column temperature and flow rate. After the proper
column was selected, each of these variables had to be optimized to achieve the best possible separation.

In this paper, each parameter was varied over a wide range of values (mobile phase concentration: 0.005–0.15N; column temperature: 55–65°C; eluent flow rate: 0.6–1.0 mL/min), while keeping all other parameters constant. The value of the parameter under study that provided the optimum resolution and analysis time was then used in the study of the next parameter, and so on, until all parameters were adjusted to their optimum values. Therefore, the optimum values used in further work were chosen by a compromise between resolution and analysis time. The analyses by HPLC were performed at 65°C under isocratic conditions. The mobile phase consisted of a 0.01N H₂SO₄ solution that was filtered through a 0.45 mm Millipore membrane (Milford, MA) and degassed by sonication for 10 min before use. The flow rate was 0.8 mL/min and the injection volume was 20 mL.

**Preparation of sample solutions**

**Preparation of mobile phase**

Sulfuric acid (0.01N) was used as the HPLC mobile phase. In a 2 L volumetric flask, 2.00 mL standardized 10N sulfuric acid was added and the volume was brought to 2 L with HPLC grade water. The solution was filtered through a 0.45 μm membrane filter and degassed before use.

**Preparation of standard solution**

One gram of pure substance was weighed accurately and added to a 50 mL volumetric flask, where it was dissolved and diluted with mobile phase. A certain volume of the solution was diluted to 250 mL with mobile phase, mixed well and filtered through a 0.45 μm membrane filter.

**Preparation of sample solution**

Samples were prepared by reacting 40% nitric acid and 40% acetaldehyde at a molar ratio of 1:0.8 at 40°C for 4 h. Then, 1 mL of sample solution was withdrawn, diluted to 250 mL with mobile phase and filtered through a 0.45 μm membrane filter.

**Results and Discussion**

**Qualitative test**

The system under investigation was an aqueous solution of the reaction mixture produced by oxidation of acetaldehyde with nitric acid. There were eight primary peaks in the chromatogram. A theoretical analysis of the reaction system showed that acetic acid, glyoxylic acid, oxalic acid and glycolic acid were produced during the oxidation, in addition to glyoxal (13–15). To associate the corresponding material with each chromatographic peak, HPLC–MS and GC–MS analysis methods were adopted. The HPLC–MS analysis results show that there are acetic acid, glyoxylic acid, oxalic acid, glycolic acid and glyoxal in the oxidation solution. The GC–MS analysis shows that butanedione exists in the reaction solution. Figure 1 shows a representative chromatogram of the sample. In the chromatogram, the peak before the oxalic acid peak was a system peak due to the variations in the concentration of H₂SO₄.

The chromatogram was compared with those of solutions of pure oxalic acid, glyoxylic acid, glyoxal, glycolic acid, and acetaldehyde. Figures 1 and 2 show that, under the selected conditions, the retention times of most peaks corresponded to those of the pure compound(s). To illustrate this point, Figure 1 shows the chromatogram of the real reaction mixture produced by oxidation of acetaldehyde with nitric acid, and Figure 2 shows the chromatogram of a synthetic standard solution prepared from the eight pure components.

**Calibration, detection limits and quantification limit**

Quantification was conducted by the use of external standard calibration curves. Five aqueous solutions, covering a broad range of concentrations, were used to plot the calibration
Table I
Calibration Curve Characteristics, LOD and LOQ

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Number of points</th>
<th>Range of peak area (10^4)</th>
<th>Concentration range (mg/L)</th>
<th>Linear regression equation</th>
<th>R^2</th>
<th>LOD (mg/L)</th>
<th>LOQ (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxalic acid</td>
<td>5</td>
<td>[0.13; 1.29]</td>
<td>[97.54; 980.76]</td>
<td>y = 130.55x + 407.51</td>
<td>0.999</td>
<td>0.10</td>
<td>0.35</td>
</tr>
<tr>
<td>Glyoxylic acid</td>
<td>5</td>
<td>[0.13; 1.38]</td>
<td>[96.12; 874.77]</td>
<td>y = 158.45x – 56.99</td>
<td>0.998</td>
<td>0.12</td>
<td>0.39</td>
</tr>
<tr>
<td>Glyoxal</td>
<td>5</td>
<td>[0.14; 1.42]</td>
<td>[37.25; 389.04]</td>
<td>y = 364.39x + 979.57</td>
<td>0.999</td>
<td>0.06</td>
<td>0.21</td>
</tr>
<tr>
<td>Glycolic acid</td>
<td>5</td>
<td>[0.09; 1.01]</td>
<td>[72.49; 725.13]</td>
<td>y = 142.17x – 1197.4</td>
<td>0.996</td>
<td>0.18</td>
<td>0.61</td>
</tr>
<tr>
<td>Formic acid</td>
<td>5</td>
<td>[0.08; 0.77]</td>
<td>[102.32; 1072.63]</td>
<td>y = 75.00x + 174.6</td>
<td>0.999</td>
<td>0.33</td>
<td>1.09</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>5</td>
<td>[0.16; 0.99]</td>
<td>[104.82; 1050.66]</td>
<td>y = 94.13x – 47.26</td>
<td>0.9978</td>
<td>0.34</td>
<td>1.13</td>
</tr>
<tr>
<td>Acetaldehyde</td>
<td>5</td>
<td>[0.071; 0.75]</td>
<td>[77.63; 783.25]</td>
<td>y = 96.66x – 735.41</td>
<td>0.9998</td>
<td>0.34</td>
<td>1.12</td>
</tr>
</tbody>
</table>

curve for each compound. Duplicate injections were made for each standard solution. The standard sample used in this study was prepared from a mixture of pure oxalic acid, glyoxylic acid, glyoxal, glycolic acid, acetic acid, acetaldehyde and formic acid. Because the butanedione concentration in the oxidation mixture was very low, butanedione was omitted from the standard sample. Standard calibration solutions were prepared from this standard solution and diluted with mobile phase by factors of 10, 30, 50, 80 and 100. HPLC analysis of each calibration solution was performed, and the calibration curves of every component were established using the peak area (y) versus the component concentration (x). Linearity was determined by constructing seven calibration curves (these plots are shown in Supplementary Figures 1–7), each using the external standard method at five concentration levels, as shown in Table I. The calculated correlation coefficients (R^2) were 0.9992 or better for every compound under the established chromatographic conditions.

In qualitative analysis, the limit of detection (LOD) is an important parameter. The LOD represents the smallest concentration that can be detected, but not necessarily exactly quantified. In chromatography, the LOD is the injected amount that results in a peak at a signal-to-noise ratio (S/N) of 3.

The limit of quantitation (LOQ) of an individual analytical procedure is the lowest amount of the analyte in a sample that can be quantitatively determined with suitable precision and accuracy. It is generally determined by analyzing samples with known concentrations of the analyte and establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision. For chromatographic methods, the LOQ can also be determined by comparing the measured signals from samples with known low concentrations of the analyte with those of blank samples. This establishes the minimum concentration at which the analyte can be reliably quantified. A typical S/N ratio is 10:1.

For the analytical method established in this paper, the LOD and LOQ for each substance are shown in Table I.

**Precision and recovery**

To judge the described method, the precision and recovery were determined by experiment. The precision was investigated six times on the same day and under the same experimental conditions for each standard calibration solution. The relative standard deviation (RSD) was in the range of 0.69–3.68% (the data are listed in Supplementary Table I). These data revealed that the method had an acceptable degree of precision.

Recovery tests were conducted to further investigate the accuracy of the method. Recovery was investigated by the standard additions method. The resultant samples were then analyzed with the method established in this paper. With the results, the component mass detected was compared with the known mass added. The recovery of the method was in the range of 98.85–106.5%, as shown in Table II.

**Robustness**

The robustness of an analytical procedure has been defined by the International Conference on Harmonization (ICH) as a “measure of its capacity to remain unaffected by small, but deliberate variations in method parameters” (16). The most important aspect of robustness is to develop methods that allow for expected variations in method parameters (17). For the determination of a method’s robustness, many method parameters, such as pH, flow rate and column temperature, were varied within a realistic range and the quantitative influence of each variable was determined. If the influence of the parameter was within a previously specified tolerance, the parameter was said to be within the robustness range of the method.

In this work, the robustness of the method was determined by making slight, deliberate changes to chromatographic conditions. Table III shows the experiments performed for the robustness evaluation and the results obtained, relative to the percentage of recovery. As shown in the table, the recovery remained within the range of 94–108%. These data were subjected to an analysis of variance (ANOVA) test to determine any significant difference between the data sets. No significant (p < 0.05) difference in mean assay was found, because the calculated value of F was lower than the critical value of F. Therefore, small variations in the chromatographic parameters did not cause significant changes in the recovery values.

In addition, the inter-day robustness evaluation was conducted and the data are listed in Table IV. No marked changes...
were observed in the chromatogram, which suggests that the developed method is robust.

Conclusions
In the production of glyoxal, the qualitative and quantitative analysis of the reaction mixture has been a long-standing problem. This paper describes a simple and rapid method for analyzing the composition of the aqueous reaction mixture produced by the liquid phase oxidation of acetaldehyde using HPLC with RID. Quantitative analysis showed that the reaction mixture produced by the liquid phase oxidation of acetaldehyde was composed of glyoxal, acetaldehyde, acetic acid, formic acid, glyoxylic acid, oxalic acid, butanedione and glycolic acid; this is very important for reaction mechanism analysis and optimization of process conditions. Compared with the conventional chemical analysis methods, the method established in this paper can considerably shorten the analysis time and improve the efficiency and accuracy of the analysis. It is especially suitable for industrial laboratories, where large sets of samples of similar origin have to be analyzed on a routine basis. In addition, this method can be applied to glyoxal production by means of other synthetic routes.

References
2. Edelkraut, F., Brockmann, U.; Simultaneous determination of carboxylic acids and carbonyl compounds in estuaries by HPLC; Chromatographia, (1990); 30:432–435.
3. Glaze, W.H., Koga, M., Cancilla, D.; Ozonation byproducts. 2. Improvement of an aqueous-phase derivatization method for the detection of formaldehyde and other carbonyl compounds formed by the ozonation of drinking water; Environmental Science and Technology; (1989); 23:838–847.
7. Hotanahall, S.S., Chandalia, S.B.; Oxidation of acetaldehyde to glyoxal by nitric acid; Journal of Applied Chemistry and Biotechnology; (1972); 22:1243–1252.
13. Hotanahalli, S.S., Chandalia, S.B.; Oxidation of acetaldehyde to glyoxal by nitric acid; *Journal of Applied Chemistry and Biotechnology* (1972); 22: 1243–1252.


