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Determination of carbonyl functional groups in bio-oils by potentiometric titration: the Faix method

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Abstract:	Carbonyl compounds present in bio-oils are known to be responsible for bio-oil property changes upon storage and during upgrading. Specifically, carbonyls cause an increase in viscosity (often referred to as 'aging') during storage of bio-oils. As such, carbonyl content has previously been used as a method of tracking bio-oil aging and condensation reactions with less variability than viscosity measurements. Additionally, carbonyls are also responsible for coke formation in bio-oil upgrading processes. Given the importance of carbonyls in bio-oils, accurate analytical methods for their quantification are very important for the bio-oil community. Potentiometric titration methods based on carbonyl oximation have long been used for the determination of carbonyl content in pyrolysis bio-oils. Here, we present a modification of the traditional carbonyl oximation procedures that results in less reaction time, smaller sample size, higher precision, and more accurate carbonyl determinations. While traditional carbonyl oximation methods occur at room temperature, the Faix method presented here occurs at an elevated temperature of 80 C.
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TITLE:

Determination of carbonyl functional groups in bio-oils by potentiometric titration: the Faix method

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pyrolysis, bio-oil, analysis, analytical, carbonyl, titration

SHORT ABSTRACT:

Here we present a potentiometric titration technique for accurately quantifying carbonyl compounds in pyrolysis bio-oils.

LONG ABSTRACT:

Carbonyl compounds present in bio-oils are known to be responsible for bio-oil property changes upon storage and during upgrading. Specifically, carbonyls cause an increase in viscosity (often referred to as 'aging') during storage of bio-oils. As such, carbonyl content has previously been used as a method of tracking bio-oil aging and condensation reactions with less variability than viscosity measurements. Additionally, carbonyls are also responsible for coke formation in bio-oil upgrading processes. Given the importance of carbonyls in bio-oils, accurate analytical methods for their quantification are very important for the bio-oil community. Potentiometric titration methods based on carbonyl oximation have long been used for the determination of carbonyl content in pyrolysis bio-oils. Here, we present a modification of the traditional carbonyl oximation procedures that results in less reaction time, smaller sample size, higher precision, and more accurate carbonyl determinations. While traditional carbonyl oximation methods occur at room temperature, the Faix method presented here occurs at an elevated temperature of 80 °C.

INTRODUCTION:

While pyrolysis bio-oils are comprised of a large variety of compounds and chemical functional groups, quantification of carbonyl groups is especially important. Carbonyls are known to be responsible for the instability of bio-oil during both storage[1] and processing[2]. The titration method presented here is a simple technique which can reliably quantify the total carbonyl content of bio-oils. Only aldehyde and ketone functional groups are quantified using this method; carboxylic acid and lactone groups are not quantified.

For analysis of bio-oils, quantification of carbonyl groups by titration has traditionally been accomplished using the method of Nicolaides[3]. This method has been commonly used in the bio-oil literature[4-7]. This is a simple procedure where carbonyls are converted to the corresponding oxime (see Figure 1). The liberated HCl reacts with pyridine to force the equilibrium to completion. The conjugate acid of pyridine is titrated with a known amount of NaOH (base titrant). The number of equivalents of NaOH used is stoichiometrically equivalent to the moles of carbonyl present in the bio-oil.

The Nicolaides method, however, has several limitations. It can require reaction times in excess of 48 hours to reach completion. This severely limits sample throughput. It utilizes pyridine, which is toxic. Sample weights of 1 to 2 g are required. Sample weight used is dependent on the amount of hydroxylamine HCl present and the carbonyl content of the sample. If initial estimates of the sample weight used are incorrect, the titration has to be repeated.

Faix et al.[8] developed a method that has been modified here to address the issues of the Nicolaides method. The reaction is carried out at 80 °C for 2 hours, thereby increasing sample throughput. Pyridine has been replaced with triethanolamine, which is a less toxic chemical. The sample size can be reduced to 100 to 150 mg. The triethanolamine consumes the liberated HCl, driving the reaction to completion and the unconsumed triethanolamine is titrated directly. A secondary titration of the hydroxylamine is unnecessary. Comparison of these titration methods has shown that the Nicolaides method significantly underestimates carbonyl content of bio-oils[9].

The method described here has been modified from the original method[8] to be more applicable to the analysis of pyrolysis bio-oils. This method was developed for the analysis of raw pyrolysis bio-oils, but it has been successfully applied to other types of biomass-derived oils, including hydrotreated bio-oils. Additionally, this method has been used to monitor changes in carbonyl content during both aging and upgrading.

PROTOCOL:

Caution: Please review all relevant material safety data sheets (MSDS) before beginning. Ethanol is flammable. All applicable chemical handling procedures should be followed, as well as all applicable waste disposable and handling procedures.

1. **Reagent Solutions**

1.1) Prepare the hydroxylamine hydrochloride solution (Solution A): Add 7.7 g of hydroxylamine hydrochloride and 50 mL of deionized water to a 250 mL volumetric flask. When all solids have dissolved, dilute to mark with ethanol. This results in a 0.55 M hydroxylamine hydrochloride solution in 80% (v/v) ethanol.

1.2) Prepare the triethanolamine solution (Solution B): Add 17.4 mL of triethanolamine to a 250 mL volumetric flask. Add 10 mL of water, and then dilute up to the mark with ethanol. 95% ethanol can also be used if the addition of water is skipped. This results in a 0.48 M triethanolamine solution in 96 % (v/v) ethanol

1.3) Prepare the hydrochloric acid solution. Either purchase 0.1 N solution or prepare using 10 mL concentrated HCl and 1 L water.

2. Bio-oil Sampling and Handling

2.1) Make sure the oil sample is at room temperature prior to withdrawing a sample. Thoroughly homogenize (mix by shaking vigorously for at least 1 minute, and visually inspect the sample to ensure it is homogenous. Some bio-oils may require longer shaking times) bio-oil to obtain a representative sample.

2.2) For the bio-oil sample, use 100 to 150 mg of bio-oil.

2.3) Minimize exposure to oxygen and heat to prevent sample degradation prior to analysis.

3. Analytical Procedure

3.1) **Standardization of the Base Solution**

3.1.1) Dry Na_2CO_3 primary standard in an oven at 105 °C overnight to ensure a dry sample. Allow the Na_2CO_3 to cool to room temperature before weighing.

3.1.2) Weigh 100 to 150 mg of sodium carbonate to a titration vessel, record the actual weight, add a stir bar and add enough water to cover the pH electrode bulb and junction.

3.1.3) Transfer the sample to a titration vessel, washing the reaction vial several times separately with ethanol and water in proportions to make a final 80% solution of ethanol/water. Titrate with the acid solution using an automatic titrator to the endpoint. Record the endpoint. The endpoint is defined as the inflection point on the titration curve.

3.1.4) Repeat the process twice to obtain three points.

3.1.5) Use the average value as the normality of the acid solution.

3.2) **Preparation of Titration Blanks**

3.2.1) For Blank A: add 0.5 mL dimethyl sulfoxide (DMSO) to a 5 mL vial with a spin vane.

3.2.2) Add 2 mL hydroxylamine hydrochloride solution (solution A).

3.2.3) Add 2 mL triethanolamine solution (solution B).

3.2.4) Cap tightly, place in preheated (80 °C) heater block or water bath and stir for 2 hours.

3.2.5) Titrate with acid solution using an automatic titrator to the endpoint, and record endpoint.

3.2.6) For Blank B: If mineral acid is suspected to be present in sample, add 0.5 mL DMSO to 5 mL vial with a spin vane.

3.2.7) Add 2 mL triethanolamine solution (solution B).

3.2.8) Cap tightly and stir at 80 °C for 2 hours.

3.2.9) Transfer the sample to a titration vessel, washing the reaction vial several times separately with ethanol and water in proportions to make a final 80% solution of ethanol/water. Titrate with the acid solution using an automatic titrator to the endpoint. Record the endpoint.

3.2.10) Repeat process three times to obtain three points.

3.3) **Validation of the Method Using a Known Carbonyl**

3.3.1) Weigh ~ 100 mg of 4-(benzyloxy)benzaldehyde (4-BBA) to a 5 mL vial, record the actual weight, add a spin vane.

3.3.2) Add 0.5 mL DMSO.

3.3.3) Dissolve the sample in 2 mL hydroxylamine hydrochloride solution (solution A).

3.3.4) Add 2 mL triethanolamine solution (solution B).

3.3.5) Close lid tightly and stir at 80 °C for 2 hours.

3.3.6) Transfer the sample to a titration vessel, washing the reaction vial several times separately with ethanol and water in proportions to make a final 80% solution of ethanol/water. Titrate with the acid solution using an automatic titrator to the endpoint. Record the endpoint.

3.3.7) Repeat the process three times, to obtain three points.

3.4) **Analysis of Bio-oil Using the Method**

3.4.1) Weigh close to 100 mg of the bio-oil to 5 mL vial, record the actual weight and add a spin vane.

3.4.2) Add 0.5 mL DMSO.

3.4.3) Dissolve the sample in 2 mL hydroxylamine hydrochloride solution (solution A).

3.4.4) Add 2 mL triethanolamine solution (solution B).

3.4.5) Close lid tightly and stir at 80 °C for 2 hours.

3.4.6) Transfer the sample to a titration vessel, washing the reaction vial several times separately with ethanol and water in proportions to make a final 80% solution of ethanol/water. Titrate with the acid solution using an automatic titrator to the endpoint. Record the endpoint.

3.4.7) Repeat the process three times, to obtain three points.

3.4.8) Blank C: If mineral acid is suspected to be present in sample, weigh close to 100 mg of the bio-oil into a 5 mL vial, record the weight and add a spin vane.

3.4.9) Add 0.5 mL DMSO.

3.4.10) Add 2 mL triethanolamine solution (solution B).

3.4.11) Cap tightly and stir at 80 °C for 2 hours.

3.4.12) Transfer the sample to a titration vessel, washing the reaction vial several times separately with ethanol and water in proportions to make a final 80% solution of ethanol/water. Titrate with the acid solution using an automatic titrator to the endpoint. Record the endpoint.

3.4.13) Repeat process three times to obtain three points.

4. Calculations

4.1) **Standardization of the Base Solution**

4.1.1) Calculate the concentration of the acid solution (mol/L) using the following equation. The weight of the dry sodium carbonate in grams is w_1, the purity is written as a fraction (i.e., 99% is 0.99), and the endpoint is in mL.

$$[acid] = (1000 * w_1 * purity\ of\ \mathrm{Na2CO3})/(105.9885 * endpoint)$$

4.2) **Validation of the Method Using a Known Carbonyl**

4.2.1) Calculate the concentration of 4BBA (mol/L) in the sample using the following equation. The weight of the 4BBA in grams is w_2, the 4BBA purity is written as a fraction (i.e., 99% is 0.99), the concentration of the acid solution (mol/L) is [acid], the triethanolamine/hydroxylamine•HCl blank endpoint is EP_{BA} (the average value of three blanks, in mL), and the endpoint is EP (in mL).

$$[4BBA] = \left(\frac{(EP_{BA} - EP)}{w_2 * purity\ of\ 4BBA}\right) * [acid]$$

4.3) **Analysis of Bio-oil**

4.3.1) Calculate the concentration of carbonyls in bio-oils [CO] (mmol/g-bio oil) using the following equation. The weight of the bio-oil in grams is w_3, the concentration of the acid solution (mol/L) is [acid], the triethanolamine/hydroxylamine•HCl blank endpoint is EP_{BA} (the average value of three blanks in mL), and the endpoint is EP (in mL).

$$[CO] = \left(\frac{(\mathrm{EP}_{BA} - EP)}{w_3}\right) * [acid]$$

4.4) **Acid Correction**

Note: The presence of mineral acids or organic acids with pKa < 2 can cause artificially low carbonyl values due to the reaction of the acid with triethanolamine.

4.4.1) If this is suspected, perform the blanks detailed in sections 3.2.6 and 3.4.8. The weight of bio-oil in the sample in grams is w_3, EP is the endpoint of the sample and EP_{BA} is the triethanolamine/hydroxylamine blank. EP_{BB} is the endpoint of Blank B, EP_{BC} is endpoint of Blank C and the weight (g) of oil used in Blank C is w_{BC}:

$$[CO_{corrected}\,] = \left(\frac{(EP_{BA} - EP)}{w_3} - \frac{EP_{BB} - EP_{BC}}{w_{BC}}\right) * [acid]$$

For bio-oil samples that contain mostly acetic acid, this is an unnecessary step.

REPRESENTATIVE RESULTS:

A typical titration curve consists of a single endpoint, as shown in Figure 2. Typical titrations for both a raw bio-oil sample, and a blank titration, are shown. As the endpoint lies at the inflection point in the titration curve; the endpoint can be easily identified by plotting the first derivative of the titration curve (shown on the right axis, dpH/dV, in Figure 2). Many automatic titration systems have software that calculates the derivative of the titration curve, which is sometimes referred to as endpoint recognition criteria (ERC).

Figure 1: Oximation Reaction.

Reaction schematic showing the conversion of a carbonyl compound to the corresponding oxime.

Figure 2: Sample and Blank Titration Curves.

Representative titration curves for a raw pyrolysis bio-oil, and a blank titration. First derivatives of the titration curves, dpH/dV, are also shown.

DISCUSSION:

Representative titration curves are shown in Figure 2. A blank titration, as well as a titration for a pyrolysis oil sample, are shown. Furthermore, the first derivative of the titration curve (dpH/dV) is shown, which allows for easy recognition of the titration endpoint. The inset table on Figure 2 shows triplicate data for both pyrolysis oil and blank titrations, with average values and standard deviations. The endpoint values shown (in mL) are used in Section 4 to calculate the total carbonyl content (in mmol/g) in the pyrolysis oil sample. For the pyrolysis oil titration shown in Figure 2, the bio-oil sample mass was 0.1148 g, the acid concentration was 0.07032 mol/L, the blank endpoint was 13.041 mL, and the pyrolysis oil endpoint was 4.891 mL. This resulted in a carbonyl content of 4.992 mmol/g bio-oil.

During method development, no interferences were seen for ethyl acetate or acetic acid. We have found that the addition of alcohols causes interferences but it is dependent on chain length. The reason is as yet undetermined but may be related to solvent properties of the alcohol. It is worth noting that monosaccharides are measured using this method. The selectivity of this method was tested by using 1-butanol, 1-pentanol, tertiary butanol, 2-propanol, ethyl acetate, acetic acid, xylose and glucose as model compounds, representing alcohol, ester, carboxylic acid and carbohydrates in the bio-oil. This method has been used to reliably analyze at least 20 raw pyrolysis bio-oils, both fresh and aged up to 120 h at 80 °C. Additionally, several hydrotreated pyrolysis bio-oils have been successfully analyzed. The applicability of this method to a wide variety of bio-oil samples, combined with a high level of accuracy and reliability, may lend this method to other applications in the future. For example, carbonyl content may be used to replace viscosity in a bio-oil aging test.

In the original method, Faix et al.[8] mixed the triethanolamine and hydroxylamine hydrochloride solutions to make a stock solution. This leads to the formation of triethanolamine•HCl which will result in a measurement error. In this method, titration of the sample should be done immediately following oximation (after 2 hours of stirring). If titration is delayed following oximation, triethanolamine can form triethanolamine•HCl, which will result in an error in the measurement.

An 80% solution of ethanol is not necessary for the transfer of the sample to the titration vessel; only a final volume of 80% ethanol is needed. As a model compound, 4-(benzyloxy)benzaldehyde (4-BBA) has a sufficiently high molecular weight to use approximately the same amount (~ 100 mg) as the bio-oil sample. While we suggest using 4-BBA as the carbonyl model compound, other compounds may be used in place of 4-BBA. Finally, blanks B and C are unnecessary if only organic acids are present in the bio-oil sample.

As discussed in the introduction, the Faix method presented here has many advantages over the traditional carbonyl titration method (the Nicolaides method). Recently, a comparison of Nicolaides and Faix titration methods has shown that the Nicolaides method significantly underestimates carbonyl content of bio-oils[9]. Additionally, in 2015 an inter-laboratory study was performed on a raw pyrolysis bio-oil, and this study included both Faix and Nicolaides titration methods[10]. It was found that the Faix carbonyl method presented here was especially reliable, with < 5% relative standard deviation (RSD) variability between all participating laboratories. In comparison, the Nicolaides method showed an inter-laboratory variability of ~10% RSD. Finally, while this method was developed for the analysis of raw pyrolysis bio-oils, it has also been successfully used for carbonyl quantification in upgraded pyrolysis bio-oils.

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DISCLOSURES:

The authors have nothing to disclose.

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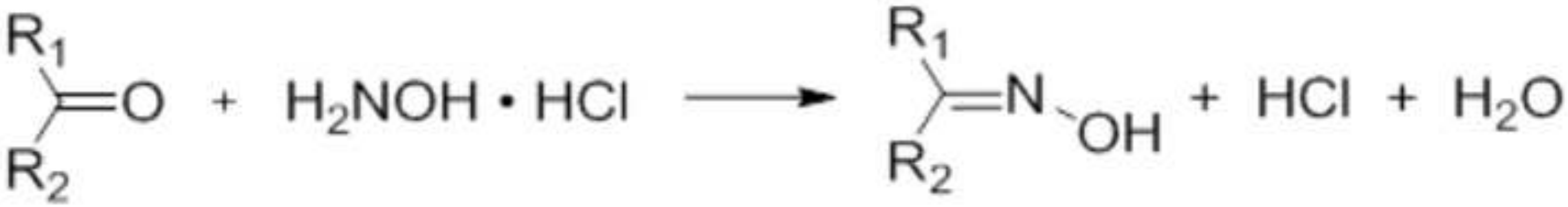

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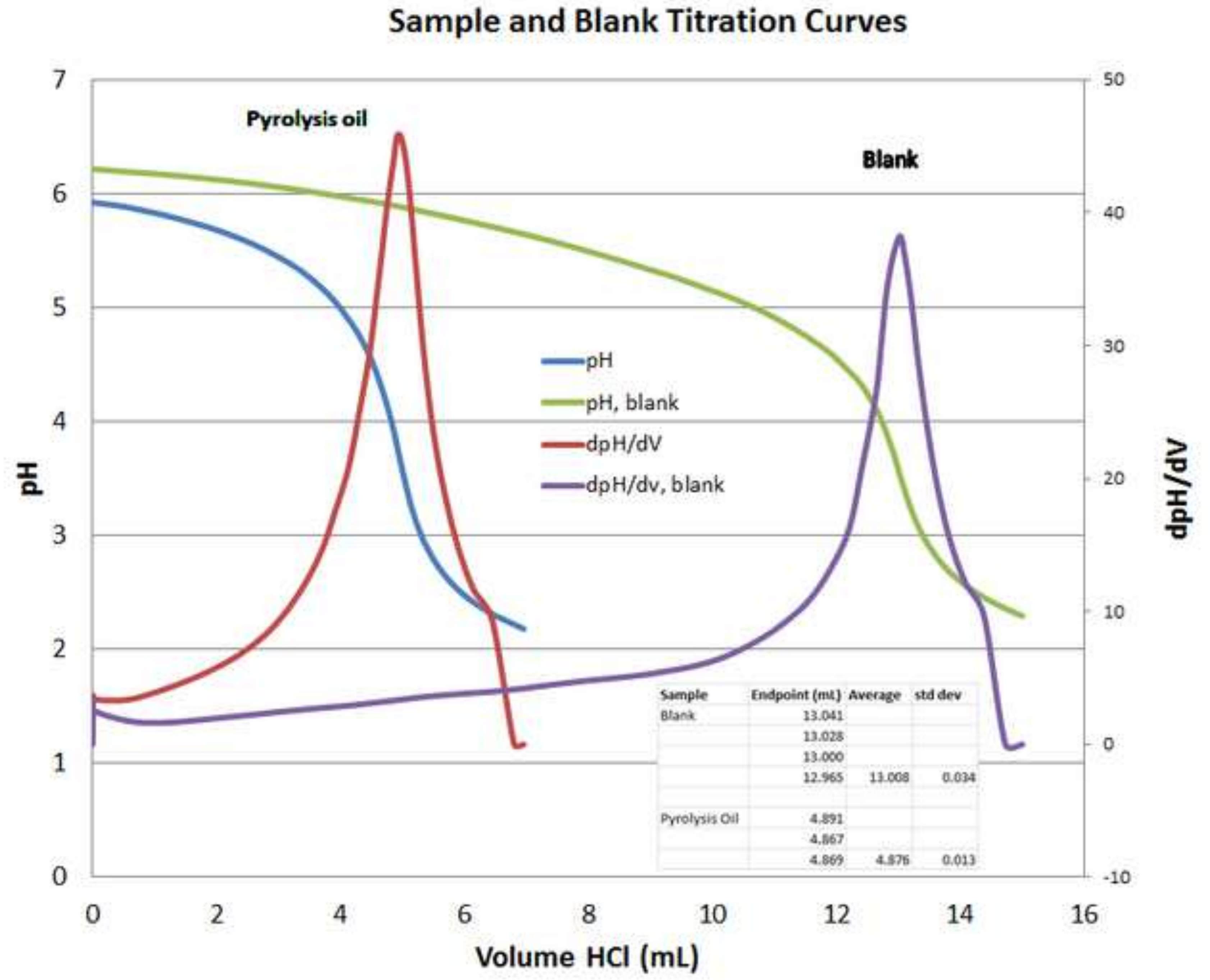

Sample	Endpoint (mL)	Average	std dev
Blank	13.041		
	13.028		
	13.000		
	12.965	13.008	0.034
Pyrolysis Oil	4.891		
	4.867		
	4.869	4.876	0.013

Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
Analytical balance			accurate to 0.1 mg
dry block heater with magnetic stirrer, or hot water bath with magnetic stirrer			
Automatic titrator			We used a Metrohm Titran
Deionized water			
Ethanol (reagent grade)			CAS # 64-17-5
Hydroxylamine hydrochloride			CAS # 5470-11-1
Triethanolamine			CAS #102-71-6
Hydrochloric acid (37%)			CAS # 7647-01-0
Sodium Carbonate (primary standard)	SigmaAldrich	223484	
4-(benzyloxy)benzaldehyde			CAS # 4397-53-9
Dimethyl sulfoxide			CAS # 67-68-5
5 mL glass Reacti-vials with solid lid and teflon spinvane	Thermoscientific	TS-13223	
200 mL volumetric flask			
Volumetric or mechanical pipettes			

do 809 automatic titrator, though other equivalent systems are acceptable

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Article Title: Determination of carbonyl functional groups in bio-oils by potentiometric titration: the Faix method.

Signature: Stuart K Black

Date: 24 June 2016

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The manuscript has been modified by the Science Editor to comply with the JoVE formatting standard. Please maintain the current formatting throughout the manuscript. The updated manuscript (55165_R1_071116.docx) is located in your Editorial Manager account. In the revised PDF submission, there is a hyperlink for downloading the .docx file. Please download the .docx file and use this updated version for any future revisions.

1. Grammar:
-3.2.9 – “th eendpoint”

<u>Response:</u> To address other comments, this sentence has been changed in the manuscript. See response to the following comment for 3.2.9.

-3.2.9, 3.3.6, 3.4.6, 3.4.12.– Please correct the run-on sentence.

<u>Responses:</u>

3.2.9: The sentence has been changed to the following: “Transfer the sample to a titration vessel, washing the reaction vial several times separately with ethanol and water in proportions to make a final 80% solution of ethanol/water. Titrate with the acid solution using an automatic titrator to the endpoint. Record the endpoint.”

3.3.6: The sentence has been changed to the following: “Transfer the sample to a titration vessel, washing the reaction vial several times separately with ethanol and water in proportions to make a final 80% solution of ethanol/water. Titrate with the acid solution using an automatic titrator to the endpoint. Record the endpoint.”

3.4.6: The sentence has been changed to the following: “Transfer the sample to a titration vessel, washing the reaction vial several times separately with ethanol and water in proportions to make a final 80% solution of ethanol/water. Titrate with the acid solution using an automatic titrator to the endpoint. Record the endpoint.”

3.4.12: The sentence has been changed to the following: “Transfer the sample to a titration vessel, washing the reaction vial several times separately with ethanol and water in proportions to make a final 80% solution of ethanol/water. Titrate with the acid solution using an automatic titrator to the endpoint. Record the endpoint.”

This change was also made for 3.1.3. Section 3.1.3 has been changed to the following: “Transfer the sample to a titration vessel, washing the reaction vial several times separately with ethanol and water in proportions to make a final 80% solution of ethanol/water. Titrate with the acid solution using an automatic titrator to the endpoint. Record the endpoint.”

-4.1.1 – Please correct the sentence structure, using imperative tense.

Response:

4.1.1: The sentence has been changed to the following: “Calculate the concentration of the acid solution (mol/L) using the following equation. The weight of the dry sodium carbonate in grams is w_1, the purity is written as a fraction (i.e., 99% is 0.99), and the endpoint is in mL.”

4.2.1: The sentence has been changed to the following: “Calculate the concentration of 4BBA (mol/L) in the sample using the following equation. The weight of the 4BBA in grams is w_2, the 4BBA purity is written as a fraction (i.e., 99% is 0.99), the concentration of the acid solution is [acid], the triethanolamine/hydroxylamine•HCl blank endpoint is EP_{BA} (the average value of three blanks, in mL) and the endpoint is EP (in mL).”

4.3.1: The sentence has been changed to the following: “Calculate the concentration of carbonyls in bio-oils [CO] (mmol/g-bio oil) using the following equation. The weight of the bio-oil in grams is w_3, the concentration of the acid solution (mol/L) is [acid], the triethanolamine/hydroxylamine•HCl blank endpoint is EP_{BA} (the average value of three blanks in mL), and the endpoint is EP (in mL).”

-Line 2.4.4 – “addition of alcohols cause”

Response: This has been changed to “addition of alcohols causes”

2. Additional detail is required:
-2.3 – Is a specific container used to achieve this?

Response: No. This is a general comment for the handling and storage of bio-oils, and is common practice.

-3.1.3 – Please describe how to “quantitatively transfer.”

Response: The statement “quantitatively transfer” has been changed to “Transfer the sample to a titration vessel, washing the reaction vial several times separately with ethanol and water in proportions to make a final 80% solution of ethanol/water.” As outlined above, this was changed for 3.1.3, 3.2.9, 3.3.6, 3.4.6, and 3.4.12.

3. Results:
-Please include a brief description in the figure legend of Figure 1.

Response: The following description was added: “Reaction schematic showing the conversion of a carbonyl compound to the corresponding oxime.”

-Please include results from a representative experiment showing that the carbonyl compounds have been quantified and the values that were obtained. These results should be discussed in the results section. This data should be supplied as a graph with appropriate error bars.

Response: Figure 2 has been revised to include the endpoint values for both blank titrations and pyrolysis oil titrations. For clarity, we have decided to only show a single representative titration curve for each, as triplicate titration curves would make the figure too confusing due to the variation in sample weight and endpoint location of the triplicates. The revised Figure 2 has a table inset showing triplicate data for both blank and pyrolysis oil titrations, with average and standard deviation values reported.

Additionally, the following text has been added in the Discussion section: "Representative titration curves are shown in Figure 2. A blank titration, as well as a titration for a pyrolysis oil sample, are shown. Furthermore, the first derivative of the titration curve (dpH/dV) is shown, which allows for easy recognition of the titration endpoint. The inset table on Figure 2 shows triplicate data for both pyrolysis oil and blank titrations, with average values and standard deviations. The endpoint values shown (in mL) are used in Section 4 to calculate the total carbonyl content (in mmol/g) in the pyrolysis oil sample. For the pyrolysis oil titration shown in Figure 2, the bio-oil sample mass was 0.1148 g, the acid concentration was 0.07032 mol/L, the blank endpoint was 13.041 mL, and the pyrolysis oil endpoint was 4.891 mL. This resulted in a calculated carbonyl content of 4.992 mmol/g bio-oil."

4. Discussion: Please discuss the limitations and future applications of the protocol.

Response: In addition to the limitations already discussed in the Discussion section, the following text has been added on line 276: "This method has been used to reliably analyze at least 20 raw pyrolysis bio-oils. Additionally, several hydrotreated pyrolysis bio-oils have been successfully analyzed. The applicability of this method to a wide variety of bio-oil samples, combined with a high level of accuracy and reliability, may lend this method to other applications in the future. For example, carbonyl content may be used to replace viscosity in a bio-oil aging test."

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
This is well written manuscript and is recommended for acceptance.

Major Concerns:
N/A

Minor Concerns:
N/A

Additional Comments to Authors:
N/A

Reviewer #2:
Manuscript Summary:
This method measure the quantity of carbonyls in the thermolysis-prepared bio-oils.

Major Concerns:
This method is quite useful to measure the chemical functionalities of bio-oil.
1. Line 100-103: Homogenizing bio-oil is not easy for a lot of "dirty" bio-oils. Shaking just for several minutes may not be enough. The use of this method may be limited for the "cleaner" bio-oils without many dirty oslid particles and tars. In many cases, the raw bio-oils exhibit the multi-phasic (more than biphasic) complex mixture. This method may be more useful for the hydrotreated or upgraded bio-oils.

<u>Response:</u> We have found this method applies to a large variety of pyrolysis bio-oils. We have tested on the order of 20 different raw pyrolysis bio-oil samples, as well as several hydrotreated samples, and found that the method worked well for all bio-oil samples tested.

The following text has been added to the Discussion: “This method has been used to reliably analyze at least 20 raw pyrolysis bio-oils, both fresh and aged up to 120h at 80°C. Additionally, several hydrotreated pyrolysis bio-oils have been successfully analyzed. The applicability of this method to a wide variety of bio-oil samples, combined with a high level of accuracy and reliability, may lend this method to other applications in the future. For example, carbonyl content may be used to replace viscosity in a bio-oil aging test.”

The homogenization instructions have been changed in 2.1 to the following: “Make sure the oil sample is at room temperature prior to withdrawing a sample. Thoroughly homogenize (mix by shaking vigorously for at least 1 minute, and visually inspect the sample to ensure it is homogenous. Some bio-oils may require longer shaking times) bio-oil to obtain a representative sample.”

2. Regarding above question, the authors did not mention the source of their bio-oil. Preparation methods and biomass resources must be mentioned.

<u>Response:</u> Details of the bio-oil used in method development are in Reference 9, but we have tested this method on a large variety of pyrolysis bio-oils, both raw and upgraded. For this JoVE manuscript, we were not aware we had to list the specifics of bio-oils tested, and as this method applies to different types of pyrolysis bio-oils, we were hoping to keep the method more general by not listing specifics of bio-oil production. Our explanation of the applicability of this method to different types of bio-oils was described in lines 74-78 in the Introduction.

Minor Concerns:
1. For the more accurated description, the names of reagents in the manuscript should be

arranged to be the same "formal" names. The authors used "ethyl alcohol" and "ethanol" at the same time.

Response: “Ethyl alcohol” at the beginning of the protocol section (Line 82) has been changed to “ethanol”.

Additional Comments to Authors:
N/A

Other changes made: Line 322 – reference 10 was updated as volume and page numbers are now available for this reference.