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Muconic acid isomers and aromatic compounds analyzed by UHPLC-DAD V.1

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https://www.nrel.gov/docs/fy19osti/74473.pdf

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We use this protocol and it's working

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Disclaimer

This protocol is for research purposes only.

Abstract

An analysis method was developed to allow for quantitation of muconic acid isomers (*cis, cis* and *cis, trans*) and aromatic compounds by ultra high pressure liquid chromatography paired with diode array detection (UHPLC-DAD). This was achieved by reproducible preparation of *c,c* and *c,t* isomers of muconic acid and chromatographic separation using a mobile phase gradient to separate various aromatic analytes and muconic isomers on a UHPLC reverse phase analytical column.

Guidelines

This protocol utilizes an ultra-high pressure liquid chromatography diode array detection (UHPLC-DAD) system manufactured by Agilent Technologies as referenced in 'Materials'. A similar chromatography and detection system can be utilized; however, some parameter nomenclature may deviate depending on the manufacturer.

Materials

Standards:

- cis cis-Muconic acid **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**15992
- Aromatics Mix Large Rev. 1 **Absolute Standards Catalog #**67185

Reagents:

- Sodium hydroxide 10N ACS reagent grade (≥30% w/w) **Fisher Scientific Catalog #** SS255-1
- Formic acid 98 % pure **Thermo Scientific Catalog #** AC147932500
- Acetonitrile Optima **Fisher Scientific Catalog #** A996SK

Equipment:

vials for isomer preparation-

Equipment	
40mL Amber Borosilicate Glass Vials	NAME
Vials	TYPE
Environmental Sampling Supply	BRAND
0040-0400-QC	SKU
https://essvial.com/product-category/glass-vialsepa-vialsvoa-vials/	LINK
40mL volume / Open-Top / Polypro with 0.125" Septa	SPECIFICATIONS

syringe filters-

Equipment

Syringe Filters, 13 mm PTFE

NAME

syringe filter

TYPE

Cytiva

BRAND

2400

SKU

https://www.cytivalifesciences.com/en/us/shop/lab-filtration/syringe-filters-non-sterile/wwptfe-non-sterile-syringe-filters/acrodisc-syringe-filters-with-universal-membranes-p-36635

LINK

13mm

SPECIFICATIONS

Equipment

syringe filters, 13mm nylon membrane

NAME

syringe filter

TYPE

Cytiva

BRAND

4550

SKU

https://www.cytivalifesciences.com/en/us/shop/lab-filtration/syringe-filters-non-sterile/nylon-non-sterile-syringe-filters/acrodisc-syringe-filters-with-nylon-membrane-p-36371

LINK

13mm

SPECIFICATIONS

guard column holder-

Equipment

SecurityGuard ULTRA holder
NAME

Guard column holder
TYPE

Phenomenex
BRAND

AJ0-9000
SKU

https://www.phenomenex.com/part?partNo=AJ0-9000
LINK

2.1 to 4.6mm ID
SPECIFICATIONS

guard column-

Equipment

UHPLC C18 guard cartridge
NAME

guard column
TYPE

Phenomenex
BRAND

AJ0-8782
SKU

https://www.phenomenex.com/part?partNo=AJ0-8782
LINK

2.1mm ID
SPECIFICATIONS

analytical column-

Equipment	
Kinetex	NAME
reverse phase analytical column	TYPE
Phenomenex	BRAND
00D-4475-AN	SKU
https://www.phenomenex.com/part?partNo=00D-4475-AN	LINK
1.7 µm / 100 × 2.1 mm / 100 Å	SPECIFICATIONS

UHPLC-DAD system-

Equipment	
1290 Infinity UHPLC	NAME
Ultra-high performance liquid chromatography system	TYPE
Agilent Technologies	BRAND
1290 Infinity UHPLC	SKU
https://www.agilent.com/en/product/liquid-chromatography/hplc-systems/analytical-hplc-systems	LINK

Troubleshooting

Safety warnings

All chemicals used for this procedure are hazardous. Read the Safety Data Sheet (SDS) for each chemical listed and follow all applicable chemical handling and waste disposal procedures. Manufacturer specific SDS information can be found by following the catalog numbers of compounds in 'Materials' list.

Before start

All solvents and chemicals used are listed in the ‘Materials’ section. These are excluded from in-line references to maintain clarity and keep the steps concise.

Preparation of Mobile Phases

1 **Mobile Phases**

1. To make the aqueous 0.2% formic acid, dilute 2.0 mL of formic acid into 1.0 L of 18.2MΩ·cm ultrapure water (UPW). Volumetric preparation is optimal.
2. Acetonitrile is used as the organic mobile phase.

Note

It is advised to prepare sufficient mobile phase for the entire analysis to reduce the need to add additional mobile phase during an active sequence. Adding mobile phase during an active sequence may cause retention time shifting if the new mobile phase varies in pH or acid concentration. This method uses 5.6 mL of mobile phase per injection, calculate how much mobile phase is needed before beginning analysis.

Preparation of Standards

2 **Standards**

This procedure for standard preparation is previously documented in our work published as the National Renewable Energy Laboratory (NREL) Laboratory Analytical Procedure (LAP) *'Determination of cis,cis-and cis,trans-Muconic Acid from Biological Conversion'* (https://www.nrel.gov/docs/fy19osti/74473.pdf). Preparation of both *cis,cis*- and *cis,trans*-muconic acid isomer standard solutions are outlined in LAP sections 10.1.3 and 10.1.4 and below in protocol step 2.1. Both standard working solutions of *c,c* and *c,t* isomers of muconic acid are prepared at 1 g/L concentrations in 0.05% v/v sodium hydroxide solution (preparation of sodium hydroxide is outlined in the referenced LAP, section 10.1.2 and below in step 2.1). Each muconic acid isomer is to be prepared in separate calibration curves in subsequent steps, the isomers should not be combined due to the tendency for *c,c* to irreversibly isomerize to *c,t*-muconic over time at room temperature.

The LAP uses the following acronyms:
c,c-muconic acid (*cc*MA)
c,t-muconic acid (*ct*MA)

2.1 *10.1.2 from LAP*
Prepare a sodium hydroxide solution (0.05% v/v) for standard preparation. Prepared by adding 66 µL 10N sodium hydroxide with an air displacement pipette to 39.934 mL of UPW measured using a repeater pipette. This solution may be scaled if necessary.

10.1.3 from LAP

Prepare the *cc*MA stock standard by weighing 40.0 ± 0.5 mg of the *cc*MA standard into a 40 mL amber vial and record the weight of the standard to the nearest 0.1 mg. Add an appropriate volume of 0.05% v/v sodium hydroxide solution using a repeater pipette to make a final concentration of exactly 1.0 mg/mL solution and mix well (vigorous shaking periodically over approximately 1 hour to allow for solubilization). The muconic acid should be completely dissolved before use otherwise the concentration of the standard will be unknown. Record the date of preparation, concentration, and any other pertinent information on the vial and store sealed at 4 °C for up to 4 months (stability study ongoing).

10.1.4 from LAP

Prepare the *ct*MA stock standard by preheating a water bath to 60 ± 3 °C. Weigh exactly 40.0 mg of the *cc*MA standard into a 40 mL amber vial (vial REQUIRED as ordered per Step 7.2.1; vial variation will lead to heat transfer difference and the reaction time will either lead to incomplete *ct*MA formation or lactone formation). Record the weight of the standard to the nearest 0.1 mg. Add 39.934 mL UPW or similar using a repeater pipette and mix well. Record the concentration of the standard, date of preparation, and any other pertinent information on the vial. Seal the standard with compatible vial top and place into the water bath so that the liquid in the vial is completely submerged for 2 hours. Shake every 15 minutes (use personal protective equipment as necessary). After 2 hours, immediately add 66 µL of 10 N sodium hydroxide using an air displacement pipette and mix. Store the sealed vial at 4 °C for up to 4 months (stability study ongoing).

2.2 Additionally, aromatic analytes compatible with this method are listed in the 'Materials' section.
Preparation of aromatic analyte standard stocks should be performed in a compatible solvent for compound solubility. A 1 g/L concentration ampule of Aromatics Mix Large Rev.1 (detailed in 'Materials') is used and diluted in UPW to reach relevant calibration curve concentrations.

2.3 **Calibration Curve**

Calibration curve preparation: aromatics and muconic acids (cis-cis and cis-trans)

Calibration level	Concentration (µg/mL) (ppm)	Volume of 1000 µg/mL working solution	Volume of UPW as diluent (µL)	Total volume (µL)
1	1	100µL of level 3 (10X)	900	1000
2	5	100µL of level 5 (10x)	900	1000
3	10	10	990	1000
4	25	25	975	1000
5	50	50	950	1000
6	75	75	925	1000
7	100	100	900	1000
8	250	250	750	1000
9	500	500	500	1000

Example calibration curve preparation (click to enlarge)

Note

Reporting limits and linear ranges may vary and should be determined for each instrument and analyte individually. The standard ranges provided by the manufacturer are suggested starting amounts and may change depending on detector response.

Preparation of Samples

3 **Samples**

- All samples containing muconic acid require a minimum 5x dilution scheme (4:1 diluent to sample ratio) for reliable quantitation of isomers due to matrix effects causing chromatographic issues. Compounds included in the aromatic mix do not require dilution without the presence of muconic acid.
- Samples must be filtered through a 0.2 µm or smaller filter prior to injection on the UHPLC.
- Samples expected to be over the linear range of the instrument should be further diluted to be within the calibration range to ensure accurate analysis and avoid carryover.

UHPLC-DAD Analysis

4 **Method Specifications**

Analysis of muconic acid isomers and aromatics is performed using an Agilent 1290 series ultra high performance liquid chromatography (UHPLC) system with a diode array detector (DAD). Complete method parameters are in the tables below.

Binary pump configuration

Flow rate	0.8 mL / min
Maximum pressure	950 bar
Mobile phase A	0.2% formic acid in UPW (v/v)
Mobile phase B	100.0% acetonitrile (v/v)

Gradient configuration

Time (min)	Composition A (%)	Composition B (%)
0.00	97.25	2.75
0.50	97.25	2.75
2.17	90.00	10.00
4.10	73.00	27.00
5.50	73.00	27.00
5.51	97.25	2.75
7.00	97.25	2.75

Column compartment parameters

Temperature	35 °C

Defined UHPLC parameters (click to enlarge)

Multisampler parameters	
Injection volume	0.5 µL
Draw speed	100 µL/min
Eject speed	100 µL/min
Wait time after draw	2 sec
Bottom sensing	yes

Diode array configuration	
Wavelength:bandwidth (reference)	210.00
	225.00
	240.00
	265.00
	280.00
	310.00
	325.00
Peakwidth	>0.013 min (20Hz)
Spectra	Store all
	190-400 nm
	2.0 nm step

Defined UHPLC-DAD parameters (click to enlarge)

Use the analytical column listed here, as well as associated guard column phase (with associated holder) listed in 'Materials'.

Equipment

Kinetex	NAME
reverse phase analytical column	TYPE
Phenomenex	BRAND
00D-4475-AN	SKU
https://www.phenomenex.com/part?partNo=00D-4475-AN	LINK
1.7 µm / 100 × 2.1 mm / 100 Å	SPECIFICATIONS

Note

Muconic acid isomers are quantified using the 265 nm wavelength and aromatics on 265 nm, 280 nm, and 310 nm. Varying wavelength signals can be used for different aromatic compounds and should be chosen at the discretion of the analyst.

Analytical Quality Control

5 Multiple strategies are utilized when performing this analysis to ensure instrument stability and reproducibility.

5.1 **Calibration Curves**

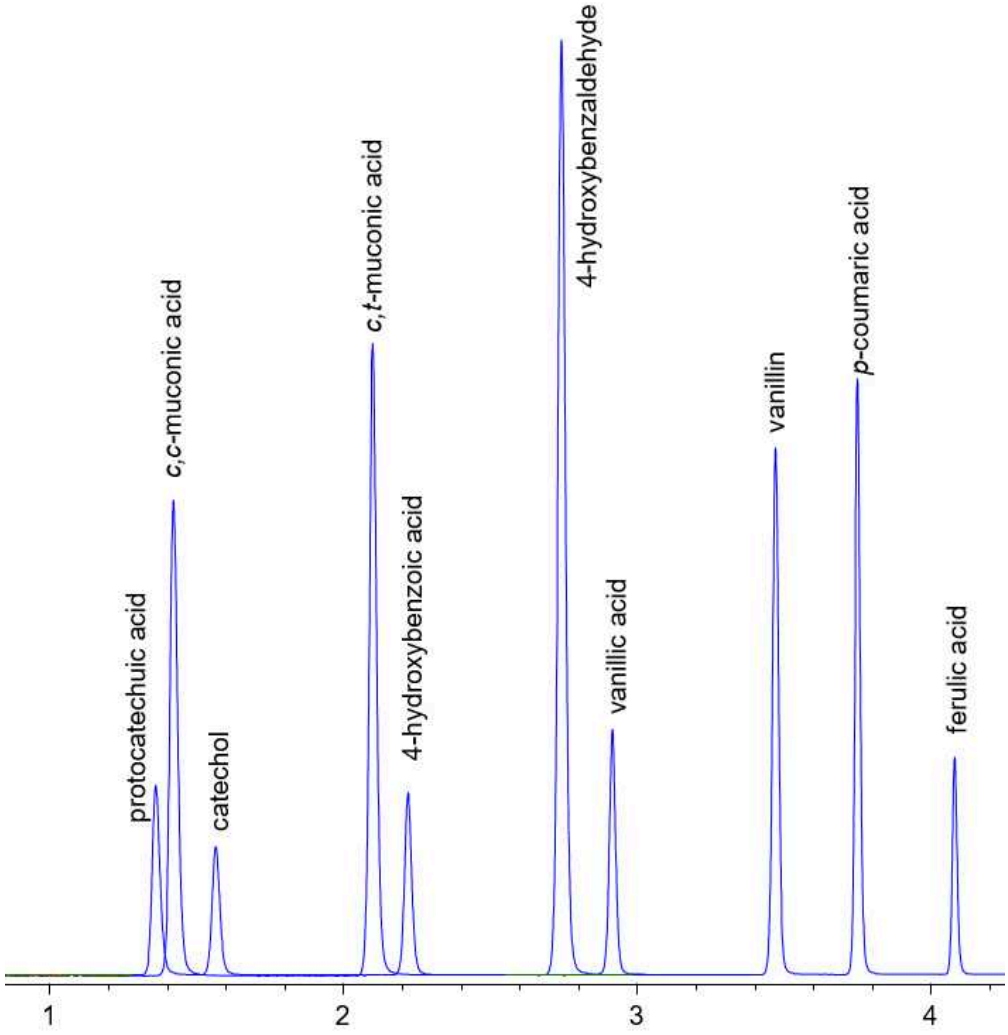
A minimum of 5 standard levels should be used.
All compounds must have a correlation coefficient (r^2) of 0.995 or greater using a linear calibration fit and ignoring the origin.

5.2 **Calibration Verification Standards (CVS)**

A calibration verification standard (CVS) is a standard from the calibration curve that is re-injected every 20 or fewer samples to ensure instrument drift remains within the determined acceptance criteria. Acceptable CVS recoveries for this analysis are within 10% of the expected amount. Acceptance criteria may differ between instruments and should be determined experimentally.

Example Chromatography

6



Elution order including muconic acid isomers and aromatic analytes analyzed using 'Aromatics Mix Large Rev. 1 Standard Mix'. Diode array detection wavelength signals are overlaid here to display analytes in elution order in one chromatogram

Data Reporting

7 Muconic acid should be reported as a sum of the two isomers. While isomerization from *c,c*-muconic to *c,t*-muconic is irreversible, environmental conditions (decreased pH, exposure to heat, etc.) may cause further isomerization of *c,c*-muconic acid to *c,t*-muconic acid. This will cause a change in the ratios of these isomers but the total muconic acid concentration will remain constant. Discrepancies in data are avoided by reporting the total of *c,c*-muconic acid plus *c,t*-muconic acid as a sum of the isomers.

Protocol references

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