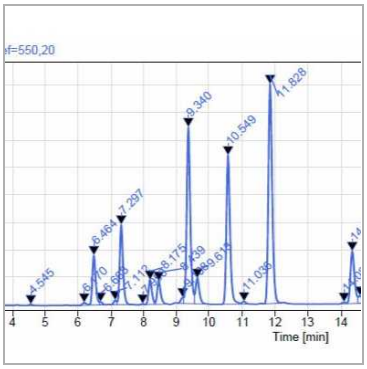

MAR 08, 2023

OPEN ACCESS

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Protocol status: In development
We are still developing and optimizing this protocol

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Carotenoid Pigment Analysis in Leaf Extracts by HPLC - UNFINISHED

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Lynn Doran
Realizing Increased Photosynthetic Efficiency (RIPE)

ABSTRACT

Pigment extraction and carotenoid analysis by high performance liquid chromatography (HPLC) from leaf tissue.

Additional References:

- García-Plazaola, J. I., & Becerril, J. M. (1999). A rapid high-performance liquid chromatography method to measure lipophilic antioxidants in stressed plants: simultaneous determination of carotenoids and tocopherols. *Phytochemical Analysis*, *10*(6), 307-313.
- Leonelli, L. (2022). Chapter Twenty - An in vivo plant platform to assess genes encoding native and synthetic enzymes for carotenoid biosynthesis. Methods in Enzymology, 671, 489-509. https://doi.org/10.1016/bs.mie.2022.03.005.
- Meuller-Moule, P., Conklin, P. L., & Niyogi, K. K. (2002). Ascorbate deficiency can limit violaxanthin de-epoxidase activity in vivo. Plant Physiology, 128(3), 970–977. https://doi.org/10.1104/pp.010924.

MATERIALS

Equipment

- HPLC, as described in before you begin.
- Microcentrifuge
- Vortex, with attached tube rack
- Tube rack
- Micropipette, 20-200 ul
- (Optional) 11mm Stainless Steel Hand Operated Crimper with Grip, ThermoFisher 60180-CR11

***Crimp top vials are used in this protocol for cost savings. If screw cap vials are used, crimper is not necessary.

Consumables and Reagents

	A	B	C	D	E	F
	Item	Vendor	Part #	Size	~ Amt/sample	Website

Keywords: HPLC, Chromatography, Carotenoids, Pigments, Plants

	A	B	C	D	E	F
	Crimp caps with septa, Aluminium,Clear PTFE/Natural Red rubber, Silver, 11 mm	VWR	46610-744A	Pack of 100	1	https://us.vwr.com/store/product/37402009/vwr-crimp-caps-for-11-mm-crimp-top-vials
	300 µl Amber Polypropylene Limited Volume Vial, 11mm Crimp/Snap Ring Top	JG Finneran Associates, Inc	30111P-1232A	case of 1000	1	https://www.novatech-usa.com/30111P-1232
	Thermo Scientific™ Target2™ Nylon Syringe Filters	Fisher Scientific	03-391-4B	Pack of 100	1	https://www.fishersci.com/shop/products/target2-nylon-syringe-filters/033914B?matchedCatNo=F2504-2&searchHijack=true&searchTerm=F2504-2&searchType=RAPID
	AIR-TITE LUER SLIP SYRINGES, 1 mL (Low Dead Space)	Air-Tite Products	MS1	Pack of 100	1 (Reusable)	https://www.air-tite-shop.com/p-16-air-tite-luer-slip-syringes.aspx?variantid=56
	Acetone, HPLC Plus, for HPLC, GC, and residue analysis, ≥99.9%	Sigma-Aldrich	650501-1L	1L	300 ul	https://www.sigmaaldrich.com/US/en/product/sigald/650501
	Acetonitrile, HPLC Plus, ≥99.9%	Sigma-Aldrich	34998-2.5L	2.5L	~40-60 mL	https://www.sigmaaldrich.com/US/en/product/sigald/34998
	Ethyl acetate, HPLC Plus, for HPLC, GC, and residue analysis, 99.9%	Sigma-Aldrich	650528-1L	1L	~20-40 mL	https://www.sigmaaldrich.com/US/en/product/sigald/650528
	Methanol, suitable for HPLC, gradient grade, ≥99.9%	Sigma-Aldrich	34885-1L-R	1L	~20-40 mL	https://www.sigmaaldrich.com/US/en/product/sigald/34885
	Tris-HCl, Molecular Biology Grade (Tris-Hydrochloride)	Promega	H5121	100 g	<0.1g	https://www.promega.com/products/biochemicals-and-labware/biochemical-buffers-and-reagents/tris_hcl_-molecular-biology-grade-_tris_hydrochloride_/?catNum=H5121
	Sodium Hydroxide (White Pellets)	Fisher BioReagents	BP359-500	500 g	<0.1g	https://www.fishersci.com/shop/products/sodium-hydroxide-white-pellets-fisher-bioreagents/BP359500

	A	B	C	D	E	F
	Gel-Loading Tips, 1-200µL	Fisherbrand	02-707-138	960 tips	2	https://www.fishersci.com/shop/products/fisherbrand-gel-loading-tips-1-200-l-4/02707138
	Micropipette tips, 20-200 ul, standard	Pipette Brand Dependent				
	1.5 mL Eppendorf Tubes	Fisherbrand	02-682-002	500	1	https://www.fishersci.com/shop/products/basix-microcentrifuge-tubes-standard-snap-caps/02682002#?keyword=02682002

To reuse 1 mL syringes:

To reuse steel grinding beads:

SAFETY WARNINGS

Uses organic solvents.

- Familiarize yourself with safety data sheets for acetone, acetonitrile, ethyl acetate, and methanol before beginning analysis.
- Wear appropriate PPE.
- Dispose of all hazardous waste according to GEGC, DRS, and UIUC guidelines.

BEFORE START INSTRUCTIONS

Collect tissue into tubes and place directly into liquid nitrogen. Grind under liquid nitrogen using tissuelyzer or mortar and pestle. This protocol assumes ~3X #7 cork borer leaf discs (ID 12mm) were collected per sample.

This protocol assumes that HPLC hardware, software, and method has already been properly configured for this analysis.

Protocol as presented is run on an HPLC (Agilent 1290 Infinity II) configured with an XSelect HSS C18 5 um VanGuard Cartridge 3.9 mm x 5 mm pre-column (Waters, 186007856) and a Spherisorb 5 um ODS1 4.6 mm × 250-mm cartridge column (PSS830615, Waters, Milford, MA) at 30°C (1290 MCT isothermal column oven, Agilent G7116B) and a vial auto-sampler (1290 Vialsampler, Agilent G7129B). A quaternary pump (Agilent 1290 Infinity II) provides a linear gradient from 100% (v/v) solvent A (acetonitrile:methanol:0.1MTris-HCl, pH 8.0; 84:2:14 [v/v]) to 100% (v/v) solvent B (methanol:ethyl acetate, 68:32 [v/v]) for 15 min, followed by 3 min of solvent B at a solvent flow rate of 1.2 mL min^{-1} to a $A_{445}$with a reference at 550 nm

by a diode array detector (1260 DAD WR, Agilent G7115A).

Software screenshots or example chromatograms are taken from OpenLab CDS Acquisition ver. 2.5 with Agilent Chemstation Integrator enabled in processing methods.

Analytical Method:

watersPIGCAR.amx

Processing Method:

pigcarprocessed_v2.pmx

NOTE:

- Sample and hardware performance can vary. Processing method is provided as a TEMPLATE ONLY and will need to be adjusted for each use case.
- This processing method does not contain calibration standards. UIUC GEGC uses external software. Calibration standards can be added to this processing method for direct analysis in OpenLab.

HPLC maintenance and storage status is maintained by the IGB GEGC Lab Supervisor. ALWAYS contact the lab supervisor before beginning analysis on the instrument. Often it may be in a storage state and will need to be changed to the appropriate chemicals and columns for analysis.

Communicate OFTEN with the lab supervisor when performing analysis.

- Immediately notify lab supervisor of any large changes in pump pressure or if pump pressure exceeds 200 bar.
- Notify lab supervisor if instrument provides any errors pertaining to any equipment (i.e. auto-sampler, pump, DAD, etc).
- Notify lab supervisor if there are any unexplained leaks on the counter or equipment.
- Notify lab supervisor if waste container has greater than 8L of waste and you are not familiar with the hazardous waste disposal protocols.
- Notify lab supervisor weekly if you are completed or if you plan to continue analysis on Monday. Communicate with lab supervisor again on Monday to see if weekly HPLC maintenance has been completed. Do not start weekly analysis until lab supervisor has confirmed that required maintenance has been completed. Lab supervisor typically tries to complete maintenance on Friday afternoons to avoid salt buildup in the instrument when it is idle over the weekend.

Prepare HPLC

1 Contact IGB GEGC Lab Manager to ensure the instrument is up to date on maintenance status, out of storage status, and in analysis mode.

Note

When the HPLC will not be used for >2 weeks, the lab manager will make sure the analytical column is rinsed with water and stored in 100% acetonitrile. The bypass column is used to fill the sample lines, gaskets, and stainless steel components with isopropanol. The lines need to be rinsed and prepared with analytical reagents prior to analysis. This is to be done by the lab manager.

Note

Due to the high concentration of Tris and the solvent changes from Tris to organic solvents, salt accumulation on the pre-column and column can occur. Coordinate with lab manager to rinse column with water at least once a week to minimize salt build up in the system and keep a low pump pressure.