Laser capture of tomato pericarp tissues for microscale carotenoid analysis by supercritical fluid chromatography

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Abstract

Plant organs and tissues are comprised of an array of cell types often superimposed on a gradient of developmental stages. As a result, the ability to analyze and understand the synthesis, metabolism, and accumulation of plant biomolecules requires improved

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methods for cell- and tissue-specific analysis. Tomato (*Solanum lycopersicum*) is the world's most valuable fruit crop and is an important source of health-promoting dietary compounds, including carotenoids. Furthermore, tomato possesses unique genetic activities at the cell and tissue levels, making it an ideal system for tissue- and cell-type analysis of important biochemicals. A sample preparation workflow was developed for cell-type-specific carotenoid analysis in tomato fruit samples. Protocols for hyperspectral imaging of tomato fruit samples, cryoembedding and sectioning of pericarp tissue, laser microdissection of specific cell types, metabolite extraction using cell wall digestion enzymes and pressure cycling, and carotenoid quantification by supercritical fluid chromatography were optimized and integrated into a working protocol. The workflow was applied to quantify carotenoids in the cuticle and noncuticle component of the tomato pericarp during fruit development from the initial ripening to full ripe stages. Carotenoids were extracted and quantified from cell volumes less than 10 nL. This workflow for cell-type-specific metabolite extraction and quantification can be adapted for the analysis of diverse metabolites, cell types, and organisms.

1. Introduction

Neighboring cell types with unique functions may have different responses to endogenous and exogenous factors, confounding the interpretation of transcriptomic, proteomic, and metabolomic studies which often use a mixture of cell types for biological sample preparation. Laser microdissection is a sample preparation tool, which enables the collection of specific cell types for extraction of biological analytes of interest. Separate collection and sample preparation of unique cell types enable high-resolution analysis of biological function that is specific to a given tissue. There is a growing body of literature concerning the application of laser capture microdissection as a single-cell-type sampling protocol in transcriptomic (Shinozaki et al., 2018), proteomic (Yang et al., 2020), and metabolomic (Fang & Schneider, 2014) studies. Tomato is a valuable fruit crop in terms of worldwide production of approximately 180 million metric tons (www.fao.org, 2019), a significant source of dietary nutrients including carotenoids, and a well-studied model system for fruit development, ripening, and quality (Fenn & Giovannoni, 2021; Giovannoni, Nguyen, Ampofo, Zhong, & Fei, 2017). A sample preparation workflow was developed to collect specific cell types from the pericarp of tomato fruit at different developmental stages and to extract carotenoid metabolites from cell samples for quantification by supercritical fluid chromatography (Fig. 1).

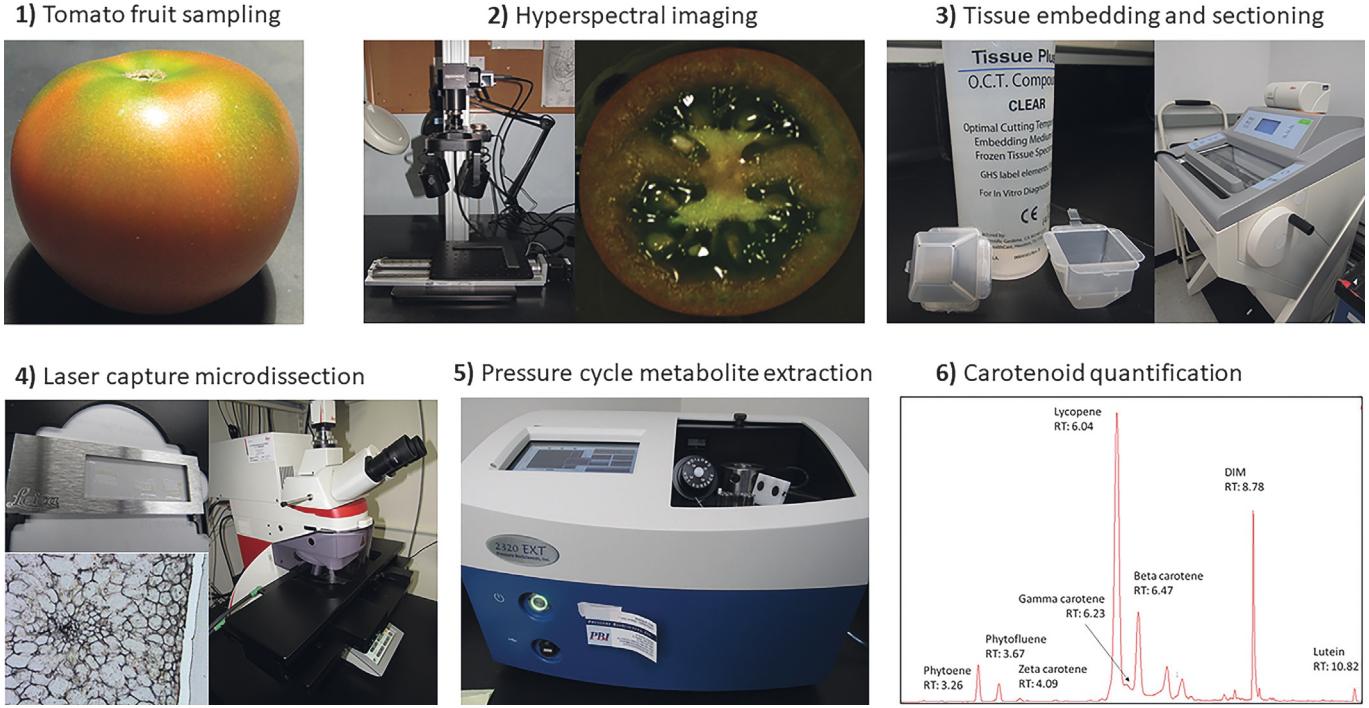

Fig. 1 Schematic of the sample preparation workflow used for cell-type-specific extraction and quantification of pericarp carotenoids during tomato fruit development. (1) Tomato fruit samples were collected from greenhouse plants at specific ripeness stages; (2) hyperspectral imaging of equatorial fruit slices was used to acquire reflectance spectra and quantify ripeness stage; (3) pericarp samples were embedded, cryosectioned, and transferred to microscope slides; (4) targeted cell types were collected from pericarp sections using laser capture microdissection; (5) carotenoids were extracted from cell samples using pressure cycling; and (6) carotenoids were quantified using supercritical fluid chromatography (see Fig. 6 for details).

1.1 Fruit developmental stage quantification using hyperspectral imaging

Hyperspectral imaging has been used for plant phenotyping in numerous contexts, including evaluation of fruit ripeness (Diago, Fernandez-Novales, Fernandes, Melo-Pinto, & Tardaguila, 2016; Rungpichayapichet et al., 2017). Machine learning algorithms applied in hyperspectral imaging data analysis select spectral features, which provide a quantitative classification of fruit ripeness state (Wei, Liu, Qiu, Shao, & He, 2014; Zhang et al., 2016). Reflectance spectra acquired from the pericarp of tomato fruit sampled throughout development can be used to quantify spectral features associated with different developmental stages and classify and group replicate samples.

1.2 Laser microdissection slide preparation

Tissue embedding and sectioning protocols previously developed for tomato fruit were adapted for laser microdissection slide preparation (Martin et al., 2016). Freshly dissected tissue is either directly immersed in embedding media or subjected to fixation and cryoprotection prior to embedding. Cryostat sectioning of embedded tissue can be performed over a range of thickness, from less than 10 μm to greater than 100 μm, with different thicknesses appropriate for different tissue types and downstream applications. The cryostat sections are transferred to laser microdissection slides, which are classified as either frame slides or glass slides. In frame microdissection slides, a thin plastic membrane comprising the center region of the slide is bordered by a metal frame. The cryostat section is affixed to the membrane, and during microdissection, the laser cuts through the membrane and the sample, which drop together into a collection tube below. In glass microdissection slides, cryostat sections are mounted on a thin plastic membrane on the underside of a glass slide, and the laser shines through the glass from above and cuts the membrane and sample, which are released together into the collection tube below.

Laser microdissection slide sample preparation is completed by fixation of the sample on the slide, washing embedding media from the sample, and dehydration of the washed slide. Depending on the chemical properties of the analytes to be extracted from collected cells, loss of analyte in these fixation, wash, or dehydration solutions is possible. The extent of analyte loss in these solutions can be evaluated by drying down and assaying their contents.

1.3 Laser microdissection sample collection

Laser function on the Leica LMD7 is optimized using several variable parameters, including power, aperture, and frequency. The efficacy of laser cutting is dependent both on the thickness and type of tissue being dissected and on the quality of the microscope slide sample preparation. The cell walls of plant tissues can be difficult for the laser to cut, particularly in thicker sections. For challenging tissue samples, the "laser screw" function on the Leica LMD7 can be used to make several subsequent cuts moving incrementally through the depth of the sectioned tissue.

Failure to thoroughly remove embedding media through slide washing will lead to poor laser cutting. In addition, slide dehydration is also critical for optimal laser cutting and sample collection, particularly with glass slides. Water can become trapped between the glass slide and the plastic membrane, leading to surface tension, which prevents separation of the sample from the slide even after complete cutting.

After cell samples affixed to the plastic membrane on the microdissection slide are cut by the laser and released from the slide, the cells and the membrane drop into collection tubes mounted on a stage below. Collection tubes can be monitored using the microscope to visually confirm that cut samples have fallen into the tube. Strategies to minimize static electricity help ensure that very small cell samples fall by gravity into their intended collection tubes. Instruments such as the Zerostat antistatic gun have been used to assist biological sample handling by dissipating static electricity (Gates & Buffington, 2011) and can be used to treat slides and collection tubes to facilitate laser microdissection sample collection. Other laser microdissection instruments use alternative capture mechanisms, and this protocol will need to be modified to match the capture mechanism of the instrument used.

1.4 Carotenoid extraction and analysis from nanoliter scale samples

Improved extraction of carotenoids from plant cell samples has been reported using a range of sample preparation strategies, including enzyme digestion, high-pressure extraction, and solvent optimization (Saini & Keum, 2018). Digestion of plant cells with enzymes such as pectinase and cellulase can improve carotenoid extraction efficiency by breaking down the cell wall matrix and releasing metabolites trapped therein (Strati, Gogou, & Oreopoulou, 2015). Improved yields of carotenoids have also

been reported using high-pressure conditions during extraction (Strati et al., 2015). Pressure cycling technology (Olszowy, Burns, & Ciborowski, 2013) has the potential to enhance both the activity of enzymes used for plant tissue digestion and the ability of organic solvents to extract metabolites from the digested sample.

Carotenoid analysis using supercritical fluid chromatography has several advantages over reversed-phase liquid chromatography, including reduced solvent usage and decreased run times (Li et al., 2015). Maximizing the proportion of the metabolite extract that can be injected onto the chromatography column is essential to enable carotenoid quantification from samples comprising small numbers of cells. This process involves determining the smallest volume possible for sample reconstitution in injection solvent, to facilitate injecting a sufficiently large fraction of the sample to ensure accurate quantification without distorting peak shape or compromising chromatographic resolution. The protocol outlined below incorporates these insights toward enhancing carotenoid extraction efficiency from nanoliter scale samples.

2. Materials, equipment, and reagents

2.1 Materials

1. All tomato fruit samples were from genotype Ailsa Craig (AC). Tomato plants were grown in a greenhouse using 400-W high-pressure sodium lights on cloudy days and to extend the day length for a photoperiod of 16 h light, 8 h dark. The temperature was kept between 25 and 27 °C during the day and between 18 and 20 °C at night.

2.2 Equipment

1. Benchtop hyperspectral imaging system (Resonon, Inc.): Pika hyperspectral imaging camera, linear translation stage, mounting tower, lighting assembly, and SpectrononPro Version 3.0.3 software
2. Fruit dissection equipment: glass plate (28 cm × 22 cm), disposable razor blades
3. Fruit sample embedding equipment: liquid nitrogen, small Styrofoam cooler, forceps, Peel-A-Way truncated T-12 embedding molds (Polysciences, Inc.), and borosilicate glass beakers (7 cm × 5 cm)
4. Stratagene UV Stratalinker 1800 UV-C (254 nm) cross-link chamber (Agilent Genomics, Inc.)

5. Polyethylene terephthalate (PET) membrane (1.4 μm) frame slide (Leica Microsystems GmbH)
6. Slide boxes for storage and slide jars for fixation, washing, and dehydration
7. Frame slide support (Leica Microsystems GmbH)
8. Cryostat Microm HM550 (Thermo Fisher Scientific)
9. Fine tip paintbrushes
10. LMD7 laser microdissection system (Leica Microsystems GmbH)
11. Zirconia/silica disruption beads, 2.3 mm diameter (Research Products International)
12. Milty Zerostat 3 antistatic gun (Millipore Sigma)
13. Barocycler model 2320 EXT (Pressure Biosciences Inc.) with two sample cartridges each holding eight polycyclohexylenedimethylene terephthalate (PCT) vials
14. Barocycler consumables: PCT vials, PCT vial caps (short), and micropestles
15. Centrivap Concentrator SpeedVac (Labconco Corporation)
16. Hamilton syringe, 250 μL (Hamilton Company)
17. Acquity UPC2 (Ultra Performance Convergence Chromatography) system with photodiode array (PDA) detector (Waters Corporation)
18. Chromatography column: Viridis HSS C18 SB (Selectivity for Bases) column, 3.0 × 100 mm, 1.8 μm (Waters Corporation)

2.3 Reagents

1. Embedding media (OCT): Tissue-Plus Optimal Cutting Solution (Thermo Fisher Scientific)
2. Nanopure water
3. Poly-L lysine solution, 0.1% in water (Millipore Sigma)
4. Slide fixation and dehydration reagent (70% ethanol, 30% nanopure water)
5. Cell wall digestion enzyme buffer: ammonium acetate, 100 mM, pH 5.0
6. Viscozyme cellulolytic enzyme mixture, 3.8 U/μL (Millipore Sigma)
7. Pectinex pectinase enzyme mixture, 0.1 U/μL (Millipore Sigma)
8. Magnesium carbonate solution in nanopure water (0.3 g/mL)
9. Tetrahydrofuran, stabilized with 250 ppm butylated hydroxytoluene (BHT)
10. Quantofix Peroxide 25 semiquantitative test strips (Machery-Nagel)

3. Protocols

3.1 Hyperspectral imaging of tomato fruit samples

1. Harvest tomato fruit samples and record the fruit ripeness stage. In the data presented, we specifically analyzed breaker fruit showing the first blush of color/carotenoid accumulation in addition to fruit 2 and 7 days post breaker where the latter have achieved full red color (Fig. 2).
2. Cut tomato fruit in half along the equatorial plane, and then cut one equatorial section (5 mm thickness) from each half of the fruit (Fig. 2).
3. Place the two equatorial fruit sections on a black aluminum foil coated tray (12 cm × 8 cm), with the fruit surfaces representing either side of the equatorial plane facing up.
4. Turn on hyperspectral imaging system and allow lights to warm up for 10 min.
5. Apply "Remove Dark Current" function in SpectrononPro software with lens cap on

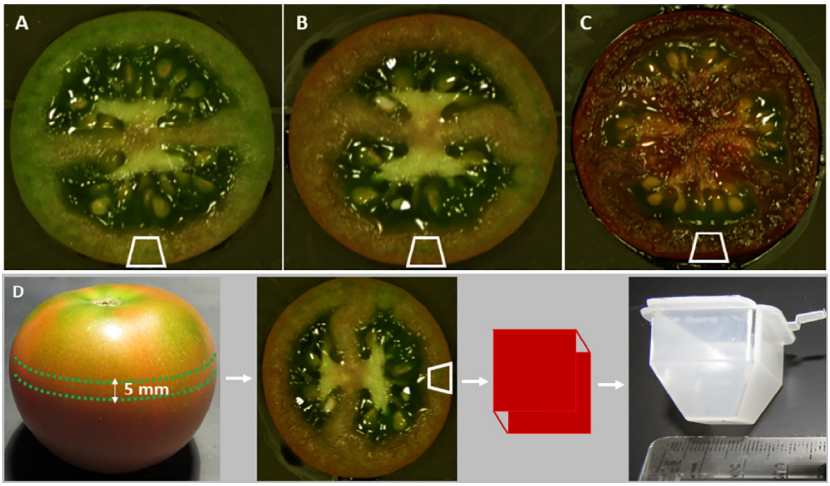

Fig. 2 Hyperspectral imaging overview scan of the three fruit samples used in this analysis, and schematic of fruit dissection and tissue embedding workflow. A pericarp region used for embedding and sectioning is indicated in the *white box*. Fruit developmental stages are (A) breaker, (B) 2 days post breaker, and (C) 7 days post breaker. (D) Fruit dissection and tissue embedding workflow: a 5-mm-thick equatorial fruit section is cut, and from this section, a tissue piece $>1\,cm^2$ is cut and placed at the bottom of an embedding mold.

6. Remove lens cap and apply "Set Reflectance Reference" function in SpectrononPro with a white reference Teflon board on the imaging stage.
7. Apply "Scan" function in SpectrononPro software and save resulting overview image file.
8. Use the lasso tool to select the region of interest (ROI) from the overview image of the fruit section for spectral acquisition. The selected ROI for each acquired spectra is half of the pericarp from one section (four spectra per fruit).
9. Export hyperspectral image data as text files recording average reflectance over the ROI at wavelengths between 385 and 1028 nm (Fig. 3).

3.2 Cryo-embedding of tomato pericarp samples

1. Pour liquid nitrogen into a small styrofoam cooler to a depth of 2–3 cm, and place four glass beakers in the cooler. Add additional liquid nitrogen as needed to keep cold throughout protocol.
2. Use a new disposable razor blade to dissect a 1 cm^2 piece of pericarp tissue from the 5 mm thick equatorial section. Dissect two pieces of

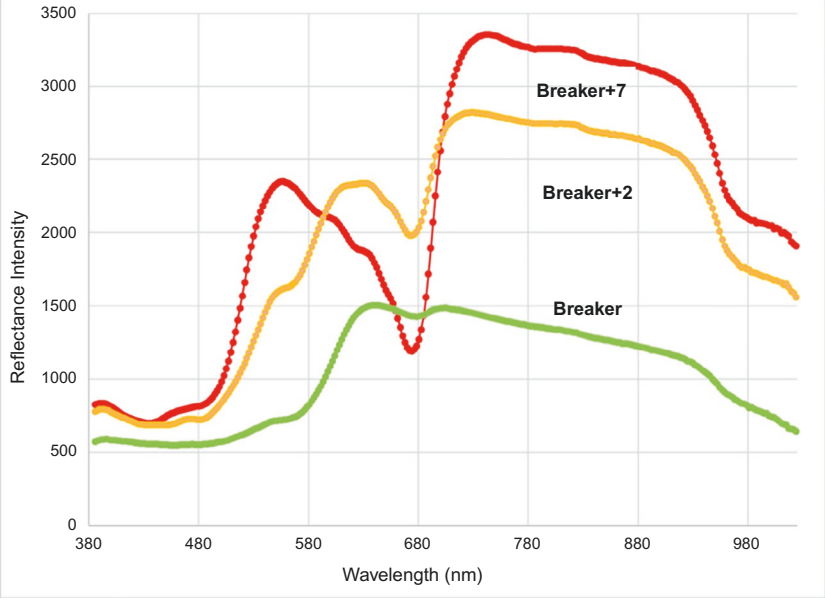

Fig. 3 Pericarp reflectance spectra for the three fruit samples used in this analysis: breaker (*green* spectrum), 2 days postbreaker (breaker+2, *orange* spectrum), 7 days postbreaker (breaker+7, *red* spectrum).

pericarp tissue, from the region midway between two pericarp-septa junctions, from each equatorial section. From each piece of fruit, dissect a total of four pericarp tissue pieces from two equatorial sections (Fig. 2).

3. Use forceps to transfer pericarp tissue into an embedding mold.
4. Squeeze optimal cutting temperature (OCT) solution over tissue in embedding mold, until OCT level reaches fill line. Push tissue down to the bottom of the mold using forceps.
5. Place each embedding mold containing tissue and OCT into a cold beaker in liquid nitrogen for 5 min, until the color of the OCT surface becomes white. Transfer frozen-embedded samples in molds to −80 °C freezer for long-term storage prior to cryosectioning.

3.3 Cryosectioning of tomato pericarp samples

1. Incubate PET frame slides in a UV-C (254 nm) cross-link chamber and deliver at least 1 J of energy (15–30 min full power) to sterilize slide and enhance adhesion between the slide and the sample.
2. Coat PET surface with 350 μL 0.1% poly-L lysine solution. Pour off excess solvent and dry slides in fume hood.
3. Load a new razor blade into the cryostat. Prechill slides and fine-tipped paintbrush in a cryostat chamber (−20 °C) for 20 min. Keep the frame slide support at room temperature outside the cryostat. Keep embedded samples on dry ice.
4. Peel away embedding mold and mount OCT-embedded sample block onto the prechilled cryostat specimen disc using room temperature OCT to adhere block to the specimen disc.
5. Set cryostat to 30 μm section thickness to trim the face of OCT block to fully expose the tissue surface (three trim cuts of 30 μm each).
6. Set cryostat to cut 70 μm thick sections and lower antiroll plate onto cutting surface so that the section slides between cutting surface and antiroll plate.
7. Turn wheel on the side of cryostat to make sectioning cut—the sample on chuck moves 70 μm forward and then down onto a razor blade on the forward edge of cutting surface.
8. Lift antiroll plate while using a fine-tipped paint brush to prevent section from rolling up.
9. Press room temperature frame slide support into the recessed back of a cold PET frame slide and gently lower slide onto 70 μm section lying on cutting surface until section adheres to PET surface. Frame slide support assists in adhesion by melting frozen OCT onto PET surface.

10. Place slide containing mounted 70 μm tomato pericarp section into a prechilled slide box on dry ice. Slides can be kept in an −80 °C freezer until ready for laser microdissection.

3.4 Laser microdissection of tomato pericarp cells

1. Slide fixation, washing, and dehydration should be done immediately before laser microdissection. Prechill 70% ethanol and nanopure water at 4 °C in slide jars.
2. Gradually warm slides from −80 to 4 °C. Transfer the slide box from −80 °C freezer to −20 °C freezer for 15 min, and then to 4 °C cold room for 15 min.
3. Slide fixation: dip slide into 70% ethanol in a slide jar for 20 s.
4. Slide washing to remove OCT: slowly dip slide into and out of nanopure water in a slide jar, five times.
5. Slide dehydration: dip slide into 70% ethanol (same slide jar as fixation) for 20 s. Dry down 70% ethanol and retain for carotenoid analysis to assess metabolite loss from the sample during slide processing.
6. Dry slides in fume hood for 5–10 min.
7. Turn on laser microdissection system.
8. Use Zerostat antistatic instrument to reduce static electricity on the slide surface and PCT vial collection tubes, to facilitate the capture of microdissected cells.
9. Mount slide into a slide holder with sample facing down. Load PCT vials into collection stage.
10. Image samples using 5× objective and calibrate laser using LMD7 software. Laser settings optimized for cutting 70 μM tomato pericarp cells with 5× objective: Power = 60; Aperture = 20; Speed = 7; Balance = 17; Line Spacing = 5; Head Current = 100%; Frequency = 120; Offset = 84. Routine preventative maintenance is performed on the laser microdissection system to ensure proper functioning of the instrument.
11. In LMD7 software, use mouse or stylus to select cells for collection. Use LMD7 software to activate laser cutting of selected region. Cut cells fall into PCT vial below. Area of cut cells is recorded.
12. Remove PCT vial from the collection stage and centrifuge at 15,000 RPM for 2 min at room temperature to pellet cells. Store cells in PCT vial at −80 °C or move directly to carotenoid extraction from cells.

3.5 Carotenoid extraction of tomato pericarp cells

1. Prepare cellulase/pectinase digestion solution:
   - 30 μL digestion solution per sample: 27 μL digestion buffer (0.1 M ammonium acetate, pH 5.0), 1 μL pectinase (3.8 U/μL), and 2 μL cellulase (0.1 U/μL).
2. Add 30 μL digestion buffer to each cell sample in PCT vial and centrifuge sample (10 s, 13,200 RPM/16,000 × *g*, room temperature).
3. Add micropestle to PCT vial and load vials into barocycler cartridges. Each barocycler cartridge holds eight PCT vials, and two cartridges are loaded into the barocycler together, allowing 16 extractions to be performed simultaneously.
4. Run barocycler for 60 pressure cycles of 1 m each (50 s at 45,000 PSI, 10 s at atmospheric pressure, 26 °C).
5. Remove PCT vials from barocycler cartridges and centrifuge vials (5 min, 13,200 RPM/16,000 × *g*, room temperature).
6. Dry digested samples in SpeedVac (30–45 min).
7. Add 25 μL magnesium carbonate solution to dried digested samples in PCT vials and briefly vortex.
8. Add two zirconia/silica disruption beads (2.3 mm) to PCT vial.
9. Use Hamilton syringe to add 125 μL tetrahydrofuran (THF) to PCT vial, close vial with short PCT vial cap, and briefly vortex. Check THF before each use to ensure that the peroxide level is less than 1 mg/L using Quantofix Peroxid 25 semiquantitative test strips (Machery-Nagel).
10. Load PCT vials into the barocycler cartridge and run the barocycler for 10 pressure cycles of 1 m each (50 s at 45,000 PSI, 10 s at atmospheric pressure, 26 °C).
11. Remove PCT vials from barocycler cartridges and centrifuge vials (5 min, 13,200 RPM/16,000 × *g*, room temperature).
12. Use Hamilton syringe to recover 100 μL of carotenoid extract in THF without disturbing pelleted magnesium carbonate and debris, and transfer extract to a 1.5 mL microcentrifuge tube.
13. Add 100 μL of THF to the same PCT vial for a subsequent extraction from the same sample, and repeat steps 10–12.
14. Repeat step 13 and pool the three extractions from each sample into a single 1.5 mL microcentrifuge tube.
15. Dry samples in speed-vac.

3.6 Carotenoid quantification

1. Dried carotenoid extracts are reconstituted in an injection solvent of ethyl acetate containing 0.05 mg/mL DIM (diindolylmethane) as an internal standard.
2. The chromatography column is equilibrated at 1% methanol. 3 μL of the sample is injected into the Acquity UPC2 system at 40 °C and a flow rate of 1 mL/min using gradient elution with a two-component mobile phase system of supercritical carbon dioxide (SC-CO_2) and methanol (MeOH).
3. The analytes are eluted using a nonlinear concave gradient to 20% MeOH over 7.5 min. The mobile-phase composition was held constant at these values for 4.5 min. The column was then reequilibrated to initial conditions over 3 min (Yazdani, Croen, Fish, Thannhauser, & Ahner, 2021).
4. UV/vis detection of carotenoid peaks was performed on a Waters PDA detector over a range of 250–700 nm. Each of the subject carotenoids was quantified at unique wavelengths optimized for both sensitivity and selectivity.
5. The carotenoid standards were quantified according to their extinction coefficient and absorbance, and known amounts were injected to obtain peak areas. These peak areas were used as a calibration to which the compounds in the samples were quantified against at the appropriate wavelength for each compound, using the TargetLynx integration software in MassLynx 4.1 (Waters; Milford, MA).

4. Results

The sample preparation protocol was applied to quantify carotenoid levels in tomato pericarp tissues collected from Ailsa Craig fruit at three different developmental stages: breaker, breaker + 2 days, and breaker + 7 days. Overview image (Fig. 2) and acquired spectra from each fruit sample (Fig. 3) are shown. Pericarp tissue samples from one fruit at each development stage were cryoembedded. From each fruit sample, the cryostat was used to cut nine replicate sections, each 70 μm thick with an area

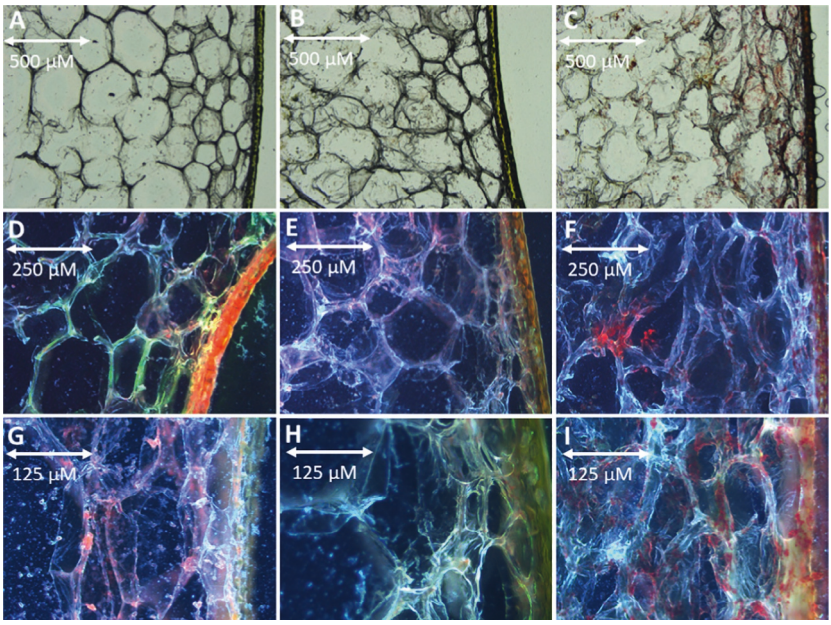

Fig. 4 Bright-field and fluorescent micrographs of 70-μm-thick cryostat sections from the three fruit samples used in this analysis. (A–C) 50 × bright-field micrographs of pericarp sections from (A) breaker, (B) breaker +2 , and (C) breaker+7 fruit. (D–F) 100 × green fluorescent micrographs of pericarp sections from (D) breaker, (E) breaker+2, and (F) breaker+7 fruit. (G–I) 200 × RGB (*red/green/blue*) fluorescent micrograph of pericarp sections from (G) breaker, (H) breaker+2, and (I) breaker+7 fruit. Scale bars are included for the size reference.

$\sim$0.5–1 cm^2, which were mounted on three replicate PET frame slides for microdissection (three sections per slide). Slides were fixed, washed, dehydrated, and imaged (Fig. 4) in preparation for laser microdissection. Two tissue regions of the tomato pericarp were collected into separate PCT vials: the cuticle and the noncuticle pericarp (containing mesocarp, exocarp, and vascular bundle) (Fig. 5).

Each replicate cuticle and noncuticle pericarp sample is comprised of all cells collected from three sections on one PET frame slide. Three replicate cuticle and three replicate noncuticle pericarp samples were collected from each fruit sample of the three developmental stages under analysis (18 total cell samples). The cell area of each sample was recorded. Carotenoid extraction and analysis were performed on the 18 samples.

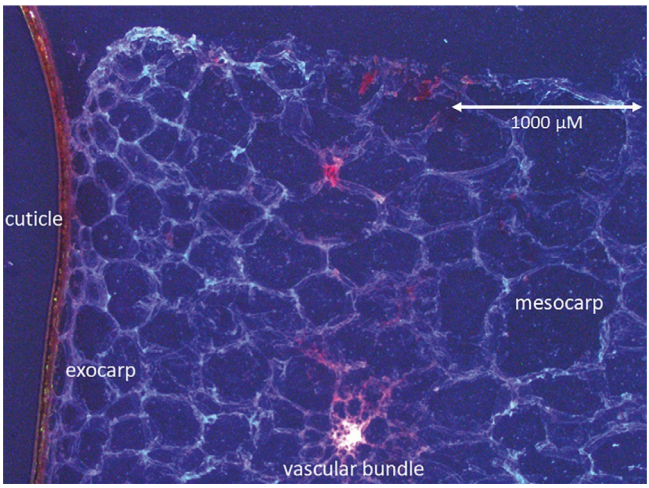

Fig. 5 Micrograph of a 70–μm-thick tomato pericarp section at 25× magnification, with RGB (*red/blue/green*) fluorescence. The positions of four pericarp cell types (cuticle, exocarp, vascular bundle, and mesocarp) are indicated. Scale bar is included for the size reference.

A full-scan (250–700 nm) chromatogram indicating the retention times for the seven carotenoids quantified in these samples, with the methanol gradient overlaid, is given in Fig. 6. Chromatograms at the λ_{max} of each carotenoid, and the UV/vis spectrum of each carotenoid, are given in Fig. 7. The concentration of the carotenoids was calculated across all extracts in relation to cell volume (Table 1; Fig. 8). While phytofluene and zeta-carotene were only detected in noncuticle pericarp samples from the breaker + 7 days stage, five of these carotenoids were detected at multiple stages, and their concentration in each tissue region is charted over the course of fruit development (Fig. 8).

The minimum starting cell volume required for carotenoid quantification was evaluated using a series of seven microdissected cuticle samples of different areas collected from breaker + 4-day stage fruit. The smallest cell sample collected had a volume less than 1 nL, for each subsequent sample the cell volume collected doubled. Two carotenoids, lycopene and beta-carotene, were quantified in samples extracted from cell volumes of 8 nL and higher (Table 2).

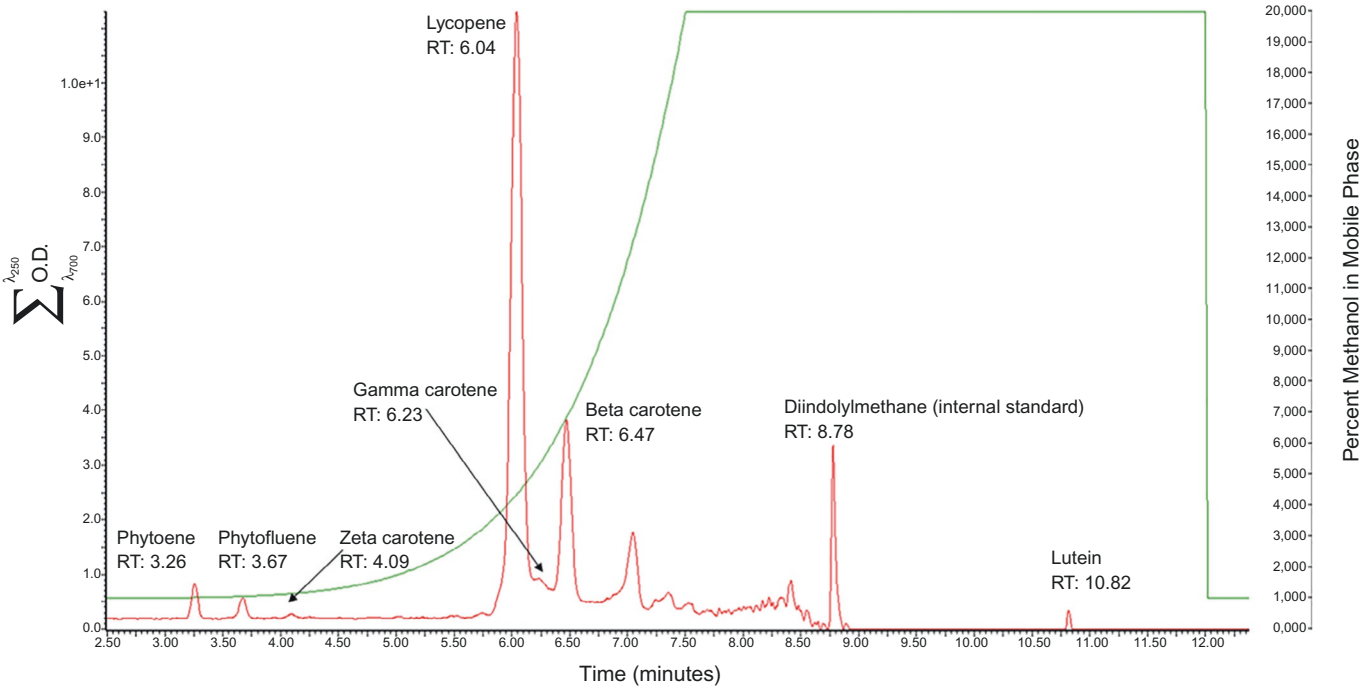

Fig. 6 Full-scan (250–700 nm) chromatogram of carotenoid extract from tomato pericarp laser microdissection samples. The *X*-axis represents the summed optical density between 250 and 700 nm. The retention time (RT) of carotenoids and the injection internal standard (diindolylmethane) are indicated. The *green curve* represents the gradient of percent methanol in the chromatography mobile phase.

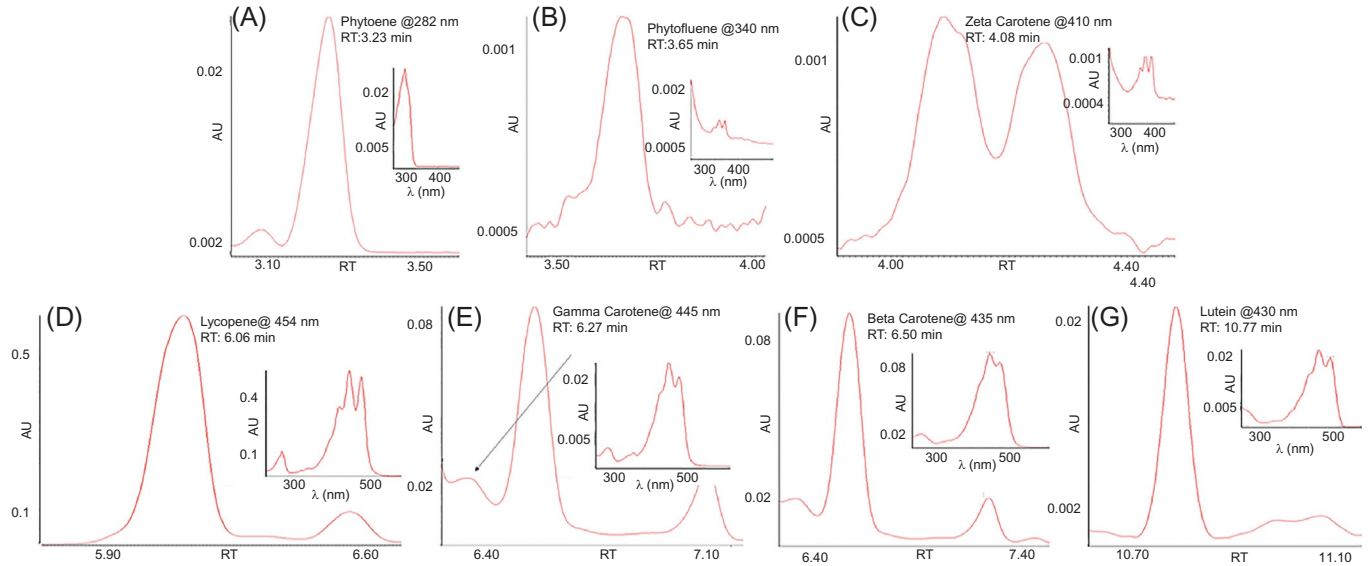

Fig. 7 Chromatograms of the seven quantified carotenoids at the wavelength indicated for each compound, zooming in on the peak at the retention time (RT) indicated for each compound. The UV/vis spectrum for each carotenoid is shown in the *inset* accompanying the chromatogram, with the λ_{max} for each compound indicated.

Table 1 Concentration of carotenoids extracted from tomato pericarp cell samples collected by laser capture microdissection.

				Carotenoid concentration (ng/μL cell volume)						
Replicate	Fruit stage	Cell type	Cell volume (μL)	Phytoene	Phytofluene	zeta-Carotene	Lycopene	gamma-Carotene	beta-Carotene	Lutein
1	Breaker	Cuticle	0.154	ND	ND	ND	36.7	0	9.03	ND
2	Breaker	Cuticle	0.142	ND	ND	ND	30.7	0	8.39	ND
3	Breaker	Cuticle	0.176	ND	ND	ND	41.9	0	9.62	ND
1	Breaker + 2	Cuticle	0.168	18.4	ND	ND	302	14.2	13.6	ND
2	Breaker + 2	Cuticle	0.187	9.05	ND	ND	203	16.5	5.85	ND
3	Breaker + 2	Cuticle	0.197	11.1	ND	ND	188	11.1	6.59	ND
1	Breaker + 7	Cuticle	0.171	22.1	ND	ND	1650	84	106	ND
2	Breaker + 7	Cuticle	0.219	7.75	ND	ND	803	20	70.2	ND
3	Breaker + 7	Cuticle	0.185	8.62	ND	ND	738	22.6	60.3	ND
1	Breaker	Noncuticle pericarp	4.96	0.2	ND	ND	6.86	0.68	7.09	0.6
2	Breaker	Noncuticle pericarp	4.33	0.41	ND	ND	12.8	1.24	13.2	0.55
3	Breaker	Noncuticle pericarp	4.60	0.23	ND	ND	7.92	0.59	7.77	0.19
1	Breaker + 2	Noncuticle pericarp	4.69	1.36	ND	ND	24.4	1.55	12.1	2.4
2	Breaker + 2	Noncuticle pericarp	4.06	1.72	ND	ND	26	1.99	12.1	2.56
3	Breaker + 2	Noncuticle pericarp	4.65	1.24	ND	ND	23.1	1.78	12.3	1.26
1	Breaker + 7	Noncuticle pericarp	4.06	4.76	0.44	0.24	243	6.31	26.5	1.89
2	Breaker + 7	Noncuticle pericarp	4.04	3.56	0.12	0.22	201	6.08	25.4	1.48
3	Breaker + 7	Noncuticle pericarp	4.01	2.27	0.14	0.09	178	4.64	28.9	1.57

ND (not determined) is indicated for samples where particular carotenoids were not quantified.

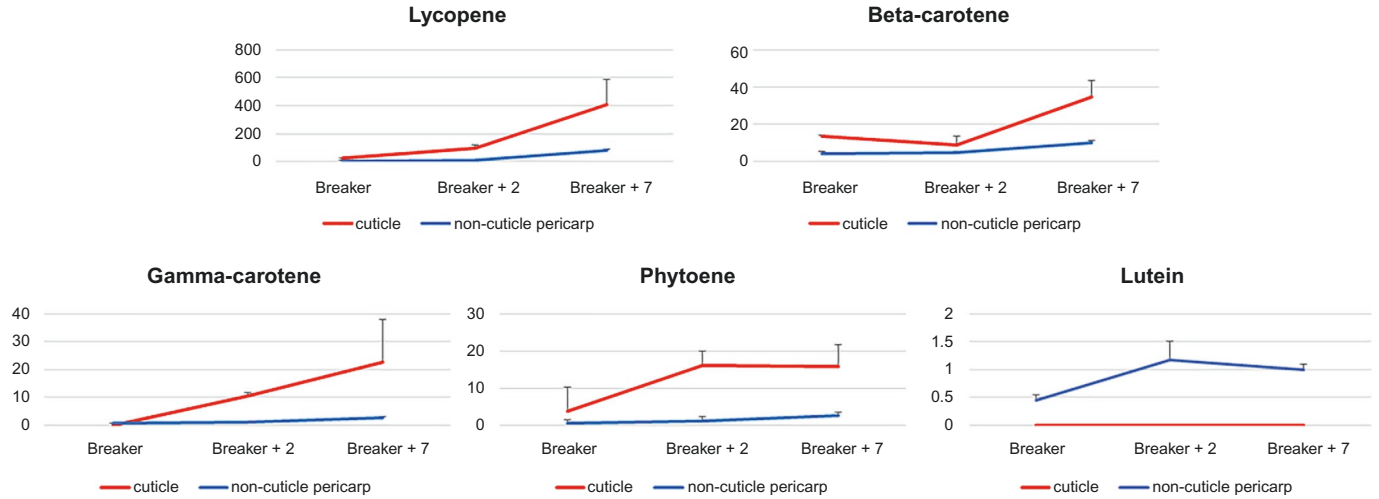

Fig. 8 Concentration (ng/μL, plus standard deviation) of five carotenoids (lycopene, beta-carotene, gamma-carotene, phytoene, and lutein) in tomato cell samples from three developmental stages (breaker, breaker + 2, and breaker + 7) and two cell types (cuticle, noncuticle pericarp).

Table 2 Limit of detection analysis of carotenoids in nanoliter volume cell samples.

Cell area (μm^2)	Cell thickness (μm)	Cell volume (μm^3)	Cell volume (nL)	beta-Carotene (ng/sample)	Lycopene (ng/sample)
9947	70	696,290	6.96	ND	ND
19,385	70	1,356,950	1.36	ND	ND
60,060	70	4,204,200	4.20	ND	ND
120,133	70	8,409,310	8.41	1.3	13.3
250,941	70	17,565,870	17.56	1.8	15.1
542,790	70	37,995,300	37.99	3.1	28.2
990,774	70	69,354,180	69.35	6.4	67.6

Cuticle cells from breaker + 4-day tomato fruit samples were collected by microdissection from pericarp sections. For each sample, cell area and volume are given along with the total mass (ng) of beta-carotene and lycopene quantified in the entirety of the sample. ND (not determined) indicates samples where beta-carotene or lycopene was not quantified.

5. Conclusions

A sample preparation protocol was developed for cell-type-specific metabolite extraction and analysis from tomato fruit samples. A carotenoid extraction method using enzyme digestion and barocycler pressure cycling was developed and applied to quantify metabolite concentration in sample volumes less than 10 nL. This sample preparation framework can be adapted for extraction and quantification of additional biological molecules, from cell samples collected by laser microdissection from alternate plant tissues and species. Determination of transcript, protein, and metabolite levels in specific tissue regions can remove confounding noise and highlight the biological processes functioning in a given cellular context.

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