**Running title: Isolation of chromoplast sub-plastidial fractions.**

**Title: Isolation and characterisation of sub-plastidial fractions from carotenoid rich fruits.**

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**Abstract**

Carotenoid biosynthesis and sequestration in higher plants occurs in the plastid organelle. Among diverse germplasm collections displaying natural variation for carotenoids and outputs from metabolic engineering experiments it has become clear that plastid type and numbers can have important implications on the quantitative composition of carotenoids accumulating. Therefore, it is important to characterise these organelles to fully evaluate the potential of the germplasm to enhance carotenoids and create nutrient dense fruits and vegetables. In this article the procedures used to isolate sub-plastidial structures from carotenoid-rich Solanaceae fruits (tomato and *Capsicum*) are described.

**1. Introduction**

Carotenoids are the largest class of natural pigments known in nature (Alcaino, et al. 2016). These pigments have roles in photosynthesis, photoprotection and general antioxidant protective mechanisms, as well as attractants for subsequent seed dispersal or pollination. Although their role as precursors to the phytohormone, abscisic acid (ABA) has been established for decades, recently other apocarotenoids have been implemented as signalling molecules and phytohormones (Al-Babili and Bouwmeester, 2015). Carotenoids are synthesised and sequestrated in plastids. In the chloroplast, carotenoids, or more precisely xanthophylls, are present in the chloroplast envelope membranes. Carotenoids are also present in the thylakoid membranes where they are associated with the photosynthetic apparatus. Plastoglobuli also contain carotenoids. Within pigmented flowers and fruits, carotenoids are found in specialised plastids known as chromoplasts. In comparison to the chloroplast, chromoplasts possess diverse internal structures, along with membranes (and/or membrane vesicles) and plastoglobuli (Egea, Barsan, Wanping, et al. 2010). In the case of *Capsicum*, chilli and sweet pepper, fruit chromoplasts typically contain fibrils, which are specialised structures that typically accumulate esterified capsanthin. Membrane structures and plastoglobuli are also present in *Capsicum* and the fibrils are believed to originate from the plastiglobuli (Berry, Rickett, Baxter, et al. 2019). In addition to diverse internal chromoplast structures, it has been observed from metabolic engineering outputs that perturb carotenoids either as; (i) ectopic carotenoid production in chloroplast-containing tissues (Fray, Wallace, Fraser, et al. 1995; Fraser, Enfissi, Halket, et al. 2007) or (ii) modifying the usual carotenoid composition of a particular tissue, which can result in altered plastid structures (Enfissi, Nogueira, D’Ambrosio, et al. 2019). This occurrence promoted the term “metabolite induced plastid differentiation (Fraser, Enfissi, Halket, et al. 2007). Subsequently this occurrence has been observed in tobacco leaves upon transient expression of the carotenoid biosynthetic enzyme phytoene synthase (Lorente, Torres-Montilla, Morelli, et al. 2019) and more active alleles of phytoene synthase in maize (Shumskaya, Bradbury, Monaco, et al. 2012). The orange (OR) gene product is associated with chromoplast development (Lu, Van Eck, Zhou, et al. 2006). However, OR is known to interact with phytoene synthase (PSY). The PSY enzyme having the highest flux coefficient in the pathway (Fraser, Romer, Shipton, et al. 2002). Therefore, it is difficult to decipher if these findings are due to the *OR* gene product exclusively or in part the action of optimal phytoene synthase activity regulated by the *OR*.

To locate pathway components/complexes within different plastid types (Wurtzel, 2019) and characterise the cellular changes resulting from altered carotenoid accumulation, it is necessary to isolate and characterise sub-plastidial fractions (Nogueira, Mora, Enfissi, et al. 2013). The present article details the procedures necessary to execute this task.

**2. Experimental approach and design**

Techniques in Molecular Biology have witnessed dramatic advances over the last decades, to the point where many of the procedures required to clone and manipulation nucleic acids use commercial kits. In contrast, many biochemical approaches are still using traditional methods with comparatively small incremental advances. The isolation of plastids (chloroplasts, chromoplasts and amyloplastids) is one example, whereby the method of choice is still predominately ultracentrifugation over continuous or discontinuous polyol gradients. The procedures described in the present article are focused on plastid isolation from Solanaceae fruits, namely tomato and *Capsicum,* which are rich in carotenoids. The procedures have been adapted from Nogueira, Mora, Enfissi, et al., 2013 and Berry, Rickett, Baxter, et al. 2019. In these fruit tissues the plastid type is a chromoplast, which are specialised plastids associated with carotenoid-accumulating tissue. The isolated tomato chromoplasts typically contain lycopene in a crystalline aggregate, and -carotene is predominantly found in the membrane and plastoglobular structures. In the case of *Capsicum* the predominant carotenoid is an esterified form of capsanthin which is packaged into fibril structures.

The procedures for isolating sub-plastidial, chromoplast structures from ripening tomato and *Capsicum* fruit are logistically challenging. Once initiated, the procedure must be completed as there are no pause points. Tissue quality is an important factor for consideration; the use of senescing fruits is not recommended, and ripening fruit should be used instead. The fruit tissue should be similar and representative of the ripening stage selected. These precise experiments require significant amounts of tissue. For example, it is ideal to have six fruit at a similar stage of development/ripening which entails growing approximately twelve plants per genotype. Routinely, our approach is to use six fruits per plant, which represents the biological replicates. It is also difficult to obtain pure fractions representing a specific sub-plastidial structure. Therefore, marker enzymes have been used to validate the sub-plastidial fractions. Recent application of “omics” technologies, such as metabolomics and proteomics, has improved measures of purity/enrichment with capability of spatial analysis.

**3. Subchromoplast fractionation of tomato and capsicum (pepper) fruit.**

**3.1. Preparation and resources.** In advance, prepare the extraction and gradient buffers (without DTT) and store at 4⁰C. Dithiothreitol is added at the point of use from a 1M stock solution (e.g., a 1000-fold dilution into the appropriate buffer volume.

1. Extraction buffer is comprised of 0.4 M Sucrose, 50 mM Tris-HCl, 1 mM DTT, 1 mM EDTA adjusted to pH 7.8.
2. Gradient buffers used are sucrose steps of

(i) 45% (w/v) sucrose, 50 mM Tricine, 2 mM EDTA, 2 mM DTT (added prior to use), and 5 mM sodium bisulphite adjusted to pH7.9 with HCl.

(ii) 38% (w/v) sucrose, 50 mM Tricine, 2 mM EDTA, 2 mM DTT (added prior to use), and 5 mM sodium bisulphite adjusted to pH7.9 with HCl.

(iii) 20% (w/v) sucrose, 50 mM Tricine, 2 mM EDTA, 2 mM DTT (added prior to use), and 5 mM sodium bisulphite adjusted to pH7.9 with HCl

(iv)15% (w/v) sucrose, 50 mM Tricine, 2 mM EDTA, 2 mM DTT (added prior to use), and 5 mM sodium bisulphite adjusted to pH7.9 with HCl

(v) 5% (w/v) sucrose, 50 mM Tricine, 2 mM EDTA, 2 mM DTT (added prior to use), and 5 mM sodium bisulphite adjusted to pH7.9 with HCl.

Fruit material is harvested the day before the preparation, the fruit (tomato and capsicum) are washed in distilled water (dH2O), deseeded, and cut into small pieces (1cm squares). Routinely, tomato ripening fruit 90 to 150 g are used, while capsicum 30 – 120 g.

**Note**: The fruit weights required depend on the colour intensity of the phenotype. For example, for red bell pepper and chilli pepper, 30 g is adequate, while yellow and orange bell pepper 100 g is recommended. Allow three hours for preparation.

The fruit weight of each batch is recorded. To reduce starch content, which is particularly important when using green fruit, fruits are stored overnight at 4⁰C in the dark prior to use. Prechilled centrifuge tubes (8 x 50 ml), centrifuge bottles (4 x 500 ml), and two glass flasks (2L) overnight at -20oC. Prechilled centrifuge rotors overnight at 4⁰C.

**3.2. Key resource table**

|  |  |  |
| --- | --- | --- |
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
| Chemicals and materials | | |
| DL-dithiothreitol (DTT) | Sigma-Aldrich | CAS 3483-12-3 |
| Sucrose | Sigma-Aldrich | CAS 57-50-1 |
| Trizma base (Tris) | Sigma-Aldrich | CAS 77-86-1 |
| EDTA | Sigma-Aldrich | CAS 60-00-4 |
| Tricine | Sigma-Aldrich | CAS 5704-04-1 |
| Cheesecloth | Holland & Barrett | Not applicable |
| Sodium bisulphite (sodium metabisulfite) | Sigma-Aldrich | CAS 7681-57-4 |
| Methanol (HPLC grade) | Fisher scientific | CAS 67-56-1 |
| Chloroform (HPLC grade) | Fisher scientific | CAS 67-66-3 |
| Experimental Models: Organisms/Strains | | |
| *Solanum lycopersicum* | Tomato Genetic Resources (www.tgrc.ucdavis.edu) |  |
| *Capsicum annuum* | www.avrdc.org |  |

**3.3. Materials and equipment**

1. Cold room (4⁰C) and freezer room (-20⁰C).
2. Centrifuges and rotors including: Sorvall RC-5C (Thermo Fisher Scientific), fixed angle rotor, GSA-3 Sorvall (Thermo Scientific), GSA-5 fixed angle rotor Sorvall (Thermo Scientific), Beckman L7 (Beckman Coulter) ultracentrifuge, SW28 swinging bucket rotor (Beckman Coulter).
3. Centrifuge bottles, 500ml (Thermo Fisher Scientific, NalgeneTM, cat# 3141-0500), centrifuge tubes, 50ml (Thick-wall Ultra Tubes) (Thermo Fisher Scientific, Nalgene™, cat# 3110-0500), centrifuge tubes, 38.5 ml (Beckman Coulter, Ultra-Clear™, cat# 344058).
4. Narrow neck conical glass flask, 5L (Fisher Scientific, Fisherbrand™, cat#11597422)\*.
5. Homogenizers: Waring blender (small laboratory blender 8010ES), (Scientific Labs, model: MIX1126). Potter-Elvehjem tissue grinder (VWR International, cat# 14231-372).
6. Fraction collector (Gilson, model: 203B).
7. Suitable ice containers
8. Plastic and glassware, funnel (glass or plastic, to fit 5 L conical flasks), glass rod and rubber teat.
9. Benchtop centrifuge (Thermo Scientific, Heraeus Pico21) and SpeedVac vacuum concentrator (Genevac EZ.27).

*Alternatives*. \*An Alternative to Glass flask conical narrow neck 5L (Fisher Scientific, Fisherbrand™, cat#11597422) is Borosilicate glass narrow neck Erlenmeyer flasks (Fischer Scientific, FisherbrandTM, cat#15479103).

**3.4. Protocol**

3.4.1. *Preparation of crude chromoplast fraction*.

1. Place fruit pieces into a prechilled blender and add extraction buffer (containing DTT, 1mM) (see section 3.1) until the solution covers the fruit. Leave the buffer to infiltrate the tissues for 5 min. This stage is best performed in the cold room and solution placed on ice.
2. Then use a rapid homogenisation from the Waring blender in a repeated manner. It is recommended for tomato fruit to use 2 x 3 sec. blasts at full power and for capsicum, 2 x 5 sec blasts. This repetitive blending will break the cell walls while the chromoplasts remain intact because of the sucrose which maintains a constant osmatic pressure.

**Note**: Where possible all procedures were performed at 4oC or placed on ice. It is important that the tissues are not over homogenized, which will lead to broken plastids and formation of gelatinous carbohydrate material.

1. Use a glass flask with funnel (stored at -20oC) to filter the slurry through 2 to 4 layers of Cheesecloth. Pass the slurry through the muslin by squeezing gently.

**Note**: It is important not to apply too much force to avoid the extrusion of polysaccharide material that will affect the pelleting of the plastid material.

1. Divide the filtered extract into two centrifuge bottles (500 ml) and add extraction buffer to the suspension until each centrifuge bottle is around two thirds full. This will generate two 500 ml centrifuge bottles per condition/genotype.
2. Balance the centrifuge bottles using a top loading balance (± 1 g) and centrifuge at 5,000 x *g* for 10 min at 4⁰C using a pre-cooled GSA-3 rotor in a RC-5C centrifuge. The step is to remove the cell debris (supernatant).
3. Carefully discard the supernatant by gently pouring from the opposite side of the pellet.
4. Leave 5 ml of the suspension in the centrifuge bottle and use this suspension to suspend the pellet. Suspend by swirling the centrifuge bottle or using a glass rod with a rubber teat.
5. Transfer the contents of the centrifuge bottle into two 50 ml centrifuge tubes and then add extraction buffer (buffer A, with DTT, section 3.1) until the tube is three quarters full. There are now four centrifuge tubes per condition/genotype.
6. Balance the centrifuge tubes (± 0.5 g) and centrifuge the tubes at 9,000 x *g* for 10 min at 4⁰C using the GSA-5 rotor in the RC-5C centrifuge.
7. Discard all of the supernatant by pouring from the opposite side of the pellet. The pellet should be silky and packed in a manner that does not allow slippage.

3.4.2. *Lysis of chromoplasts*

1. The crude chromoplast pellet is resuspended with 3 ml of the Tricine buffer containing sucrose (45% w/v; buffer B.i, see section 3.1Bi). At this point the tube contents can be vortexed in 5 sec bursts at full power, repeating five times. Again all procedures where possible are carried out on ice.
2. The resulting chromoplast suspension is poured into a Potter-Elvehjem tissue lyser, which is placed on ice. The tubes containing the lysed chromoplasts are washed by resuspension with B.i buffer (45% sucrose Tricine buffer), 0.5 ml and added to the chromoplast suspension already present in the Potter-Elvehjem apparatus.
3. The chromoplasts present in the suspension are completely broken using the Potter-Elvehjem tissue lyser by the passage of ten strokes. It is important that resistance is felt throughout this process.

## 3.4.3. *Separation of subchromoplast components using a sucrose gradient.*

1. The lysed chromoplast suspension is transferred into a falcon tube and maintained on ice. The Potter-Elvehjem lyser apparatus is rinsed twice with the B.i buffer, Tricine buffer containing 45% sucrose (5 ml) which is combined with the rest of the lysed chromoplast fraction in the cold falcon tube.
2. Concurrently, a sucrose gradient is prepared in three 38.5 ml UltraClear™ centrifuge tubes (2-3 tubes per condition). This gradient is prepared by transferring 8ml of the lysed chromoplast fraction (in buffer B.i, 45% sucrose Tricine buffer) to the bottom of the tube on which is layered: Buffer B.ii 38% sucrose Tricine buffer (6ml); then buffer B.iii, 20% sucrose Tricine buffer (6ml); then buffer B.iv, 15% sucrose Tricine buffer (4ml), and finally buffer B.v, 5% sucrose Tricine buffer (8ml).

**Note**: Add each layer, drop by drop, on the inner wall of the tube, following a circular movement. Ideally, multiple researchers should perform the task of preparing the gradients (e.g. one researcher per tube) to avoid losses due to heating. ,

1. Place the 38.5 ml centrifuge tubes in a stable metal container ready for centrifugation. Balance all tubes (including the screw caps) to ± 0.1 g, with buffer B.v containing 5% sucrose in Tricine. The tubes are placed in a prechilled SW28 rotor. Check and record their position in the rotor and centrifuge at 24, 000 rpm (100,000 x *g*) at 4⁰C for 19 hr.

3.4.4. *Fraction collection*.

1. Carefully remove tubes from the centrifuge and store the tubes on ice.
2. Photograph the gradients (ideally at 4oC. Then collect the fractions with a Gilson 203B fraction collector and pump system. Set the speed to 24.5 on the pump system (or -22.5 with anticlockwise) to collect 1 ml in a 2 ml microcentrifuge tube. Always keep the needle at the surface just under the meniscus. Fraction collection is carried out at room temperature but all solution, fractions and collection tubes are kept on ice.

**Note**: Do not collect the pellet, leave a few mL. Place the needle into the blank prechilled buffer B.v (5% w/v sucrose in Tricine) to retrieve the final fraction from the tubing.

1. Store the fractions at -20⁰C or at -80⁰C for enzyme assays or further metabolite profiling.

**Pause point:** Collected fractions can be stored until ready to extract.

**4.** **Extraction of carotenoids, isoprenoids, and proteins from sub-chromoplast fractions.**

Carotenoids and isoprenoids are extracted from the isolated fractions using the protocol described in the series Chapter “Metabolomic approaches for the Characterisation of Carotenoid Metabolic Engineering *in planta*” with some modifications. The modifications include adjustments made for the extraction from liquid fractions and the preparation of a protein fraction.

1. Selected aqueous fractions are thawed for metabolite and protein analysis in a water bath at 40oC, and 0.5 ml removed and placed in a micro-centrifuge tube (2ml). Methanol (250 μl) is added sample is vortexed for 10 sec after which Chloroform (750 μl) was added and the suspension vortexed for 10 sec. Finally, this mixture was incubated on ice for 20 min.
2. The extraction mixtures were centrifuged using a benchtop centrifuge using top speed (11180*g* for 5 min.
3. After centrifugation, a liquid/liquid partition was formed. The chloroform organic lower layer was removed and placed in a new microcentrifuge tube (2ml). A further quantity of chloroform (750 μl) was added to the remaining aqueous material and vortexed. This procedure represented a re-extraction.
4. The re-extraction mixture was centrifuged as described in step 2. The chloroform layer removed and placed in the tube with the original chloroform fraction to create a pooled chloroform extract. The pooled extract was taken to dryness using a vacuum centrifuge for 40 min and store at -20 in the dark.
5. To extract the proteins from the remaining aqueous solution, ice-cold methanol (750ml) was added to the original tube and vortex for 10 secs. The aqueous methanol extracts were centrifuged at max speed (11180*g*) rpm) for 10 min to pellet the protein fraction.
6. The supernatant was removed using a pipette with a 1ml blue tip and the protein pellets are stored at -80⁰C.

## **5. Spatial proteomic analysis and sub-chromoplast fractions.**

**5.1. Preparation and resources.** It is optimal to prepare four Sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE) prior to extraction of the fractions. SDS-PAGE with 12.5% acrylamide w/v, can be stored for up to a week at 4oC with a water-saturated butanol layer on top of the gel. The stacking gel (5% w/v acrylamide) is prepared prior to electrophoresis (Laemmli,1970).

**5.2. Key resource table**

|  |  |  |
| --- | --- | --- |
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
| Chemicals | | |
| DL-dithiothreitol (DTT) | Sigma-Aldrich | CAS 3483-12-3 |
| High-Range Rainbow Molecular weight markers | GE Healthcare | RPN75GE |
| Tris-HCl | Sigma-Aldrich | CAS 1185-53-1 |
| Water | Fisher scientific | CAS7732-18-5 |
| Glycerol | Sigma-Aldrich | CAS 56-81-5 |
| Bromophenol blue | Sigma-Aldrich | CAS 115-39-9 |
| Sodium dodecyl sulphate (SDS) | Sigma-Aldrich | CAS 151-21-3 |
| Acetonitrile (HPLC grade) | Fisher scientific | CAS 75-05-8 |
| Formic acid (HPLC grade) | Fisher scientific | CAS 64-18-6 |
| Isopropanol (HPLC grade) | Fisher scientific | CAS 67-63-0 |
| Ethanol | Fisher scientific | CAS64-17-5 |
| Glacial acetic acid | Fisher scientific | CAS64-19-7 |
| Ammonium persulphate | Sigma-Aldrich | CAS7727-54-0 |
| Glycine | Sigma-Aldrich | CAS56-40-6 |
| Ammonium bicarbonate | Sigma-Aldrich | CAS1066-33-7 |
| Iodoacetamide (IAA) | Sigma-Aldrich | CAS144-48-9 |
| -mercaptoethanol | Sigma-Aldrich | CAS 60-24-2 |
| ProteoSilver™ Plus Silver kit | Sigma-Aldrich |  |
| Trypsin (sequencing grade) | Promega | VS111 |

### **5.3 Materials and Equipment**

1. Analytical balance

2. Thermomixer (e.g. Eppendorf Thermomixer comfort), Vortex, vacuum concentrator (e.g. SpeedVac, Thermo Fischer Scientific) and standard tabletop centrifuge.

3. Short thread glass vial (2.0ml), (Agilent technologies 5182-0715), 9mm blue screw cap with PTFE/ septum, (Agilent Technologies, 5182-0717 and0.1mL glass insert with bottom spring (Supelco 854110).

4. Liquid chromatograph coupled to a mass spectrometer (e.g. Agilent Infinity 1290 with 6550 Ion Funnel QTof).

5. Gel-casting apparatus (e.g. Hoefer Scientific Instrument) and protein electrophoreses apparatus (e.g. Hoefer Scientific Instrument).

6. Eppendorf® Protein LoBind microcentrifuge tubes (0.5ml), (FisherScientific 0030108094)

and 4mm syringe filter tips, nylon, 0.22μm (e.g. Chromacol).

7., Ultrasonic bath (XUBA 3), Grant Ltd.

8. Barrier filter pipette tips (10l), Thermo Fisher Scientific, AM126035.

9. Flat-bed scanner, Image Scanner, densitometer, Amersham.

### **5.4 GEL-LC/MS Protocol**

5.4.1.  *SDS-PAGE separation and identification of sub-chromoplast proteins*.

**Note.** Fractions towards the top of the gradient contain less proteins, whereas fractions ≥15 contain more proteins and the fractions associated with the stroma contain the most proteins. Hence, adjust the dilution of proteins in loading buffer accordingly, e.g. fraction 1 with 30μL and fraction 26 with 100μL. These volumes typically result in 10g protein per lane, which is the target concentration for optimal visualisation and analysis, using a 1.0 mm gel thickness and 8 x 8.0 cm gel dimension.

1. To the protein pellets (Section 4, step 6), SDS loading buffer (30-100μL) is added and the tubes incubated at 100°C for 5 min in the thermomixer, then centrifuged for 30 sec at 11180*g* using a benchtop microcentrifuge.
2. The SDS solubilized protein samples are loaded onto a 12.5% SDS-PAGE gel and electrophoresed at 80V for ~3hrs.
3. After electrophoresis, the SDS-PAGE gel is removed and fixed in a solution of ethanol (50% v/v) and acetic acid 10%v /v). The manufacturer’s instructions for the ProteoSilverTM Plus Silver Stain Kit are used to wash, sensitize, and visualize the protein bands in the gel. A photograph of the gel is taken, or the gel is scanned using a Flat-bed scanner. The gel is then stored in ultrapure water until the trypsin digest.

5.4.2. *In-gel trypsin digest of sub-chromoplast proteins for identification using MS/MS database searching.*

**Note**. Prior to digestion the following buffers/solution should be prepared. DTT 1M stock solution stored frozen, 55mM Iodoacetamide, 50mM Ammonium bicarbonate, Sequencing grade Trypsin 0.1mg/ml in 50mM Ammonium bicarbonate.

1. Excise with a clean sterile scalpel on a glass plate, the protein bands as blocks and cut them into small cubes (~2-3 mm). Transfer the gel pieces into 0.5 mL protein lo-bind tubes and store at -20°C until the trypsin digest.
2. Add 5mM DTT (100μL) to the gel pieces and incubate at 56°C for 1hr in the thermomixer, then remove the DTT solution and replace with 55mM IAA solution (100μL). Incubate in the dark at room temperature for 45min. The IAA solution is removed and then the gel pieces are incubated with 50mM ammonium bicarbonate (100μL) for 20 min at room temperature. Repeat this step three times.
3. All liquid is removed from the gel pieces. Then add acetonitrile (100μL) and incubate at room temperature for 15 min, repeat this step three times until the gel pieces are solid and white. Remove the last solution from the gel pieces and incubate on ice for 15 min.
4. Freshly prepared trypsin solution (0.1mg/ml in 50mM ammonium bicarbonate), 10-20μL (or so that a 1:20 to 1; 50 w/w ratio is reached), is added to the gel pieces and left until the gel pieces have absorbed all of the liquid, after which 50 mM ammonium bicarbonate solution (100l) is added and incubated at 37°C for 16 hr.
5. Supernatants are then transferred to a new protein lo-bind tube, using filter pipette tips (10l), (ThermoFisher Scientific) to avoid potential contamination.
6. Acetonitrile/ultrapure water (75:25), (100μL) is added to the gel pieces and the mixture sonicated, using an ultrasonic bath for 15 min, full power. The supernatant is removed and pooled with the previous supernatant (step 5). This step is repeated three times.
7. The pooled supernatants containing the peptides from the target protein, are dried using a vacuum concentrator in “aqueous” mode for ≥ 5hrs. Samples are stored at -20°C until LC-MS analysis.

5.4.3. *LC-MS/MS separation and identification of subchromoplast proteins.*

**Note**. Upon obtaining peptides after trypsin treatment of the subchromoplast proteins, generic LC-MS protocols can be applied. This proteomic analysis can be done via service providers or specific platforms established in the laboratory. In the present case an Agilent Infinity 1290 with 6550 Ion Funnel, Quadrupole Time of flight (Qtof) is used. Therefore, generic details will be referenced accordingly.

1. Resuspend the digested protein in 40μL LC-MS grade water (40μL) and vortex for 30sec. The resuspended peptides are filtered using a 0.22μm nylon filter and the filtrate placed into a glass insert (0.1mL) positioned into a glass vial (1.5ml). These vials are placed into the autosampler, then chromatographed and analysed according to Nogueira, Mora, Enfissi, et al 2013).

5.3.4. *Data analysis for protein identification.*

The Identification of peptides and proteins from the LC-MS/MS outputs uses both the Mascot and Daemon software (Matrix Science) or Spectrum Mill (Agilent) workbenches. The Specific protein databases can be downloaded from platforms such as Sol Genomics Network (<https://solgenomics.net/>), UniProt (<https://www.uniprot.org/proteomes/>) and NCBI (<https://www.ncbi.nlm.nih.gov/protein/>). The following search settings are used routinely.

1. Enzyme specificity: trypsin, Fixed modification: Carbamidomethylation and Variable modification: oxidation of methionine and Peptide tolerance: 100 ppm and MS/MS tolerance: 50 Da.
2. Once the peptide/protein summary is created, identification is confirmed by comparing the molecular weight of the identified protein with the estimated size of of the protein targeted on SDS-PAGE. Additionally, the peptide score (≥5), protein coverage and number of peptides match to the target protein, are used as criteria for valid interpretation of protein identity. Spectrum Mill also provides a percentage of score peak intensity (≥60), which describes the quantity of the extracted MS/MS ions explained by the database hit.

**6. Expected outcomes.**

Visual inspection of the gradients generated by ultracentrifugation (section 3.4), will reveal the different subchromoplast components associated with ripening tomato and capsicum fruits. Figure 1 illustrates the outputs from ripe tomato fruit and the designation of the subchromplast fractions. Carotenoid-containing plastoglobuli are observable as the top layers of the gradient, being the least dense. More dense fractions within the mid-section of the gradient represent membranous material. Carotenoid crystals and fibrillar plastoglobuli are also expected to be located in this mid-section region. The final third of the tube, below the membrane fractions, is expected to contain the contents of the stroma and membrane proteins which have become detached in the fractionation process. The pellet, which should be low in amount, contains aggregated debris and proteins; these structures (or materials) cannot be separated on the gradient used. Overloading the gradient with too much material can result in poor resolution and contamination with nonspecific, abundant proteins. This problem is solved by reducing the weight of the starting material until the fractions between the bands and plastoglobuli are clear of colour.

Incomplete homogenization of the chromoplast suspension will result in all of the membranes and proteins pelleting to the bottom. This problem can be avoided by completely shearing the chromoplasts with the Potter-Elvehjem tissue grinder as determined by the absence of aggregates visible upon microscopic inspection.

**7. Summary and perspectives.**

In recent years, systems level analysis using “omic” technologies has routinely been adopted to characterise natural variation ([Schauer](https://pubmed.ncbi.nlm.nih.gov/?term=Schauer+N&cauthor_id=16531992), [Semel](https://pubmed.ncbi.nlm.nih.gov/?term=Semel+Y&cauthor_id=16531992), [Roessner](https://pubmed.ncbi.nlm.nih.gov/?term=Roessner+U&cauthor_id=16531992), et al. 2006) and metabolic engineering outputs (Enfissi, Barneche, Ahmed, et al. 2010). Robust methodologies to isolate subplastidial components/structures as described in the present article provide the opportunity to obtain valuable spatial data. Such spatial data in the case of carotenoid biosynthesis should provide greater understanding on how the biosynthetic pathway is controlled and regulated at a cellular level, as well as differentiating biosynthesis from sequestration. These data will augment future engineering of biology approaches directed towards the enhancement of nutritional and industrial carotenoids in plant cell factories.

**8. Figure legends**

Figure 1. Separation of subchromoplast components from ripening tomato fruit. A-Diagrammatic representation of the components and position in the gradient, B-provides a visual representation of a typical separation of subchromoplast components and C-Transmission Electron Microscopy (TEM) of a representative chromoplast showing membranes, carotenoid crystals and plastoglobuli.

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