Metabolites Measurements with the plate reader

adapted from Paris-Sud; Guillaume Queval, Graham Noctor 2007

This protocol allows you to measure 8 samples together. If Ascorbate is not measured, you can do 16 samples in < 4 hours

Equipment and Reagents

<table>
<thead>
<tr>
<th>Machine/Product</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crushed Ice</td>
<td></td>
</tr>
<tr>
<td>Centrifuge – cooled</td>
<td>Eppendorf 5415R with F45-24-11 rotor</td>
</tr>
<tr>
<td>Mixer Mill / Cryo Mill</td>
<td>Retsch mM 400 with 2x PTFE Adapter rack for 10 reaction vials 1.5 and 2.0 ml.</td>
</tr>
<tr>
<td>Microtube Vortex</td>
<td>Ika Vortex 1</td>
</tr>
<tr>
<td>Plate reader</td>
<td>BioTek PowerWave HT with Gen 5 software</td>
</tr>
<tr>
<td>Plastic UV -96-well plates</td>
<td>Greiner Bio One Cat. No.655 801</td>
</tr>
<tr>
<td>Liquid Nitrogen (+ thermos jar)</td>
<td></td>
</tr>
<tr>
<td>2-VP</td>
<td>2-Vinylpyridine 97%</td>
</tr>
<tr>
<td></td>
<td>Aldrich 132292</td>
</tr>
<tr>
<td>AO (Ascorbate oxidase from Curcubita) (1000U)</td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td></td>
</tr>
<tr>
<td>Dimethylsulfoxide</td>
<td>sigma D5879</td>
</tr>
<tr>
<td>DTNB 5,5-dithiobis(2-nitro-benoic acid) ≥ 98%; 396.35 g.mol⁻¹</td>
<td>Sigma D8130</td>
</tr>
<tr>
<td>DTT (1,4-Dithiothreitol) ≥ 99 %; 154.24 g.mol⁻¹</td>
<td>Sigma 43815</td>
</tr>
<tr>
<td>G6P</td>
<td></td>
</tr>
<tr>
<td>D-glucose 6-phosphate sodium salt</td>
<td>Sigma D7879</td>
</tr>
<tr>
<td>GR</td>
<td></td>
</tr>
<tr>
<td>Glutathione reductase from Baker's Yeast</td>
<td>Sigma G3664</td>
</tr>
<tr>
<td>GSH (L-gluthathione, reduced) &gt;98%; 307.3 g.mol⁻¹</td>
<td>Sigma G4251</td>
</tr>
<tr>
<td>GSSG (L-glutathione, oxidized) ~98 %; 612.63 g.mol⁻¹</td>
<td>Sigma G4376</td>
</tr>
<tr>
<td>HEPES</td>
<td></td>
</tr>
<tr>
<td>HEPES free acid; 238.31 g.mol⁻¹</td>
<td>Sigma H3375</td>
</tr>
<tr>
<td>Hydrochloric Acid concentrated HCl 37 %; 1.19 g.ml⁻¹; 36.46 g.mol⁻¹ = 12.08 mol.l⁻¹</td>
<td>VWR 20252.290</td>
</tr>
<tr>
<td>Na₂-EDTA (disodium salt dehydrate) 99 %; 372.2 g.mol⁻¹</td>
<td>Sigma ED2SS</td>
</tr>
<tr>
<td>NADPH &gt;98% (reduced form) tetrasodium salt</td>
<td>Apollo Scientific BIB3014</td>
</tr>
<tr>
<td>Sodium Hydroxide</td>
<td></td>
</tr>
<tr>
<td>NaOH pellets</td>
<td>Vel 1735</td>
</tr>
</tbody>
</table>
Prepare Working Reagent Solutions

2-VP: 2-vinyl-pyridine
- Always work in the flow hood and with gloves
- Store at -20°C in 1 ml aliquots

AO: 40 U/ml Ascorbate oxidase
- Prepare a 10X stock solution, use complete bottle (400 U/ml 200 mM NaH$_2$PO$_4$ (pH 5.6))
- Divide in 280 µl aliquots
- Dilute 10x in 2520 µl 200mM NaH$_2$PO$_4$ (pH 5.6)
- Divide in 250 µl aliquots (40U/ml 200mM NaH$_2$PO$_4$ (pH 5.6))
- Store at -20°C in aliquots: don’t forget to order new enzymes when almost finished

GR: 20 U/ml Glutathione reductase
- Centrifugate the complete content of the bottle during 5 min 14000 rpm at 4°C
- Remove the supernatant
- Resuspend the pellet in the correct volume of 200 mM NaH$_2$PO$_4$-EDTA (pH 7.5) for 20U/ml
- Divide in 820 µl aliquots
- Store at -20°C in aliquots: don’t forget to order new enzymes when almost finished

Following products can be stored for about ~2 months:

<table>
<thead>
<tr>
<th>Solution</th>
<th>Weight</th>
<th>Product</th>
<th>in</th>
<th>pH</th>
<th>with</th>
<th>Store</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 M HCl</td>
<td>41.46 ml</td>
<td>HCl 37.5%</td>
<td>100 ml dH$_2$O</td>
<td>-</td>
<td></td>
<td>4°C</td>
</tr>
<tr>
<td>1 M HCl</td>
<td>8.29 ml</td>
<td>HCl 37.5%</td>
<td>100 ml dH$_2$O</td>
<td>-</td>
<td></td>
<td>4°C</td>
</tr>
<tr>
<td>200mM HCl</td>
<td>8.29 ml</td>
<td>HCl 37.5%</td>
<td>500 ml dH$_2$O</td>
<td>-</td>
<td></td>
<td>4°C</td>
</tr>
<tr>
<td>5 M NaOH</td>
<td>20 g</td>
<td>NaOH</td>
<td>100 ml dH$_2$O</td>
<td>-</td>
<td></td>
<td>4°C</td>
</tr>
<tr>
<td>1 M NaOH</td>
<td>4 g</td>
<td>NaOH</td>
<td>100 ml dH$_2$O</td>
<td>-</td>
<td></td>
<td>4°C</td>
</tr>
<tr>
<td>200mM NaOH</td>
<td>3.997 g</td>
<td>NaOH</td>
<td>500 ml dH$_2$O</td>
<td>-</td>
<td></td>
<td>4°C</td>
</tr>
<tr>
<td>120mM NaH$_2$PO$_4$</td>
<td>8.25 g</td>
<td>NaH$_2$PO$_4$,2H$_2$O</td>
<td>500 ml dH$_2$O</td>
<td>7.5</td>
<td>NaOH</td>
<td>4°C</td>
</tr>
<tr>
<td>200mM NaH$_2$PO$_4$</td>
<td>13.8 g</td>
<td>NaH$_2$PO$_4$,2H$_2$O</td>
<td>500 ml dH$_2$O</td>
<td>5.6</td>
<td>NaOH</td>
<td>4°C</td>
</tr>
<tr>
<td>200mM NaH$_2$PO$_4$ + 10mM EDTA</td>
<td>13.8 g</td>
<td>NaH$_2$PO$_4$,2H$_2$O</td>
<td>500 ml dH$_2$O</td>
<td>7.5</td>
<td>NaOH</td>
<td>4°C</td>
</tr>
<tr>
<td></td>
<td>(*)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(*)Put this buffer in the fridge, do not put it on ice because it will crystallize, when crystallization occurs, just mix the solution and you can use it again

Following product can be stored 1 month:

<table>
<thead>
<tr>
<th>Solution</th>
<th>Weight</th>
<th>Product</th>
<th>in</th>
<th>Store</th>
</tr>
</thead>
<tbody>
<tr>
<td>12mM DTNB</td>
<td>47.6 mg</td>
<td>DTNB</td>
<td>10 ml DMSO</td>
<td>RT in dark</td>
</tr>
</tbody>
</table>

Note: when it turns yellow, throw it away because it is oxidized

Following products can be stored 1 week:
**Solution Weight in Vol Buffer Store**

<table>
<thead>
<tr>
<th>Solution</th>
<th>Weight</th>
<th>in Vol</th>
<th>Buffer</th>
<th>Store</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM GSH</td>
<td>~3.5 mg</td>
<td>(1000/3.073*weight) µl</td>
<td>200mM NaH₂PO₄ (pH 5.6)</td>
<td>4°C</td>
</tr>
<tr>
<td>10 mM GSSG</td>
<td>~7.0 mg</td>
<td>(1000/6.126*weight) µl</td>
<td>200mM NaH₂PO₄ (pH 5.6)</td>
<td>4°C</td>
</tr>
<tr>
<td>25 mM DTT</td>
<td>~3.5 mg</td>
<td>(1000/3.856*weight) µl</td>
<td>dH₂O</td>
<td>4°C</td>
</tr>
</tbody>
</table>

Following product can be stored 1 week although Aliquots of 1.5 ml in Eppendorf tubes can be stored for longer periods at -80°C

<table>
<thead>
<tr>
<th>Solution</th>
<th>Product</th>
<th>MW</th>
<th>Weight</th>
<th>in Vol</th>
<th>Buffer</th>
<th>Store</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mM NADPH</td>
<td>NADPH</td>
<td>833.4</td>
<td>~10 mg</td>
<td>(1000/8.334*weight) µl</td>
<td>dH₂O</td>
<td>-20°C</td>
</tr>
</tbody>
</table>

### Prepare the 1.5mL Eppendorf tubes

- You should harvest samples in 1.5 ml tubes
  - Roots: 1 sample ~50 mg
  - Leaves: 1 sample 100 mg
- Cut pH papers 4x in order to have smaller paper strips that don’t absorb too much of your sample
- Label 1.5mL eppendorf tubes (this is for 1 x 8 samples)
  - GSH1 → GSH3 to dilute the GSH stock
  - GSH0’ → GSH3’ to prepare the GSH standard
  - GSSG1 → GSSG4 to dilute the GSSG stock
  - GSSG0’ → GSSG3’ to prepare the GSH standard and incubate with 2-VP for GSSG
  - GSSG0’’ → GSSG3’’ to collect the supernatant after incubation with 2-VP for GSSG
  - 1 → 8 to collect the supernatant after centrifugation for AsA and GSH
  - 1’ → 8 ‘to collect the supernatant after centrifugation for NAD and NADP
  - 1’ → 8 ‘to collect the supernatant after centrifugation for NADH and NADPH
  - 1A → 8A to incubate the sample with DTT for total AsA
  - 1G → 8G to incubate the sample with 2-VP for GSSG
  - 1G’ → 8G’ to collect the supernatant after incubation with 2-VP for GSSG

- Put the samples in liquid nitrogen
- Keep the buffers at 4°C
- Defrost all products on ice
- Turn on centrifuge at 4°C
- Turn on the oven at 20°C
- Prepare 8 x 2 tungsten carbide beads

### Prepare a broad standard range of 8 concentrations

*GSH broad range (10µl/well for leaves and roots)*

#### Dilute 10 mM GSH stock 100X in 2 steps

- (100µM = 100pmol/µl = 1000pmol/10µl = 1000pmol/well)
- Step 1 = dilute 10X = 1mM: 450µl 200mM NaH₂PO₄ (pH5.6) + 50µl 10mM stock
- Step 2 = dilute 10X = 100µM: 900µl 200mM NaH₂PO₄ (pH5.6) + 100µl 1mM stock

#### Prepare standards

\[ 1000 \text{ pmol}^* = \quad 100\mu\text{l} \quad 100\mu\text{M GSH stock} \]
\[ 500 \text{ pmol} = \quad 100\mu\text{l} \quad 200\mu\text{M NaH}_2\text{PO}_4 \quad (\text{pH}5.6) \quad + \quad 100\mu\text{l} \quad 100\mu\text{M GSH stock} \]
250 pmol = 100µl 200mM NaH₂PO₄ (pH5.6) + 100µl 500pmol GSH
125 pmol = 100µl 200mM NaH₂PO₄ (pH5.6) + 100µl 250pmol GSH
62.5 pmol = 100µl 200mM NaH₂PO₄ (pH5.6) + 100µl 125pmol GSH
31.25 pmol = 100µl 200mM NaH₂PO₄ (pH5.6) + 100µl 62.5pmol GSH
15.625 pmol* = 100µl 200mM NaH₂PO₄ (pH5.6) + 100µl 31.25pmol GSH
[0 pmol = 100µl 200mM NaH₂PO₄ (pH5.6)]

* All standards are used the first time, but these two can be left out if everything falls within range

**GSSG broad range (20µl/well for leaves and roots)**

**Dilute 10mM GSSG stock 1000X in 3 steps**
- Step 1 = dilute 10X = 1mM: 450µl 200mM NaH₂PO₄ (pH5.6) + 50µl 10mM stock
- Step 2 = dilute 10X = 100µM: 900µl 200mM NaH₂PO₄ (pH5.6) + 100µl 1mM stock
- Step 3 = dilute 10X = 10µM: 900µl 200mM NaH₂PO₄ (pH5.6) + 100µl 100µM stock

**Prepare standards**

| 200 pmol* = | 100µl 200mM NaH₂PO₄ (pH5.6) + 100µl 10µM GSSG stock |
| 100 pmol = | 100µl 200mM NaH₂PO₄ (pH5.6) + 100µl 10µM GSSG stock |
| 50 pmol = | 100µl 200mM NaH₂PO₄ (pH5.6) + 100µl 50pmol GSSG |
| 25 pmol = | 100µl 200mM NaH₂PO₄ (pH5.6) + 100µl 25pmol GSSG |
| 12.5 pmol = | 100µl 200mM NaH₂PO₄ (pH5.6) + 100µl 12.5pmol GSSG |
| 6.25 pmol = | 100µl 200mM NaH₂PO₄ (pH5.6) + 100µl 6.25pmol GSSG |
| 3.125 pmol* = | 100µl 200mM NaH₂PO₄ (pH5.6) + 100µl 3.125pmol GSSG |
| [0 pmol = | 100µl 200mM NaH₂PO₄ (pH5.6) ] |

* All standards are used the first time, but these two can be left out if everything falls within range

**Acidic extraction protocol**

**Crushing and dividing the samples**
- Put 2 tungsten carbide beads in the 1.5 ml Eppendorf tubes and shred them ice cold (in liquid nitrogen) during 3.5 minutes at maximum amplitude. Leave the beads (tungsten carbide is non-magnetic).
  - *At this point you can freeze the samples again or you can continue with the extraction!*
- Add 800 µl (for Arabidopsis th.) or 400 µl (for Lemna m.) of 200 mM HCl to the frozen sample and vortex until it is thawed, keep the sample on ice.
- Centrifuge the samples 15 min maximum speed at 4 °C.

**Neutralization**

* Apply the same procedure for each sample, always keep the samples on ice.

- take 300µl aliquots (1 → 8) and add 30 µl 200 mM NaH₂PO₄ (pH 5.6)
- vortex and put on ice
- obtain pH 4-5 with 200 mM NaOH
  (when > pH 5 the sample gets oxidized): start with 100 µl 200 mM NaOH and vortex immediately
  measure pH by putting the pH strip in the solution
afterwards add 100, 50, 20 or 10 µl 200mM NaOH (this should be ca. 200 µl, write down the exact volume) when you passed the pH you can go back with HCl but don’t forget to write it in your notes.

**Incubate samples for oxidized glutathione measurements**
- put 100 µl extract of the ~ 300 µl aliquot (1 → 8) in 1G → 8G
- add 1.3 µl 2-VP (work in flow, use gloves!) and vortex
- add 1 µl 2-VP to the standard (GSSG0’ → GSSG3’)
- vortex and incubate 30 min at room temperature
- continue with next measurement during incubation
- centrifuge 15 min at 4 °C to precipitate 2-VP
- continue with measurements during centrifugation
- transfer 80 µl supernatant to clean tubes: 1G’ → 8G’ and GSSG0’’ → GSSG3’’
- centrifuge again 15 min at 4 °C to precipitate 2-VP

**Incubate samples for total ascorbate measurements**
- put 140 µl 120 mM NaH$_2$PO$_4$ (pH 7.5) in 1A → 8A
- add 10 µl 25 mM DTT
- add 100 µl extract of the ~300 µl aliquot (1 → 8)
- Vortex and incubate 15 min at 20 °C in the cooled microtube block
- continue with next measurement during incubation
- adjust to pH 5.5 with HCl (optimal pH for AO) and write down the exact volume

**Reduced Ascorbate measurement**
- Start your measurements with ascorbate because it is less stable
- Turn on the plate reader and prepare the runs to measure
- Take a UV 96-well plate
  - We always measure the samples in triplicate, for 8 samples you have to prepare 8 x 3 = 24 wells
- Add 155 µl diluted 200 mM NaH$_2$PO$_4$ (pH 5.6) (= 100 µl dH$_2$O +55 µl buffer) in each well using the automatic multichannel
- Vortex the extracts and add 40 µl extract to the wells (use the same tip for each triplicate)
- Put the plate in the reader and start the first measurement: M_ASCORBATE ENDPOINT this will measure the total absorbance at 265 nm after 20 sec shaking
- Use the automatic pipet and add 5 µl AO to each well (use the same tip)
- Put the plate back into the reader and start the second measurement: M_ASCORBATE KINE+AO after 20 sec shaking, ascorbate oxidation is measured, after 5-10 min the reaction should be completed. Where do the CIG intervene? When the alarm would be given

**Total Ascorbate measurement**
The protocol is exactly the same for both measuring only reduced ascorbate and total ascorbate, only for total AsA you have to incubate with DTT first in order to reduce all ascorbate that is present in the sample
Glutathione measurement

The protocol is almost the same for both measuring total GSH and only oxidized GSSG, for only GSSG you incubate with 2-VP in order to block all GSH present in the sample and add 20 µl extract instead of 10 µl

- the samples are measured in triplicate, for 8 samples you have to prepare 8 x 3 = 24 wells
- the standard are measured in duplicates, for 8 standards you have to prepare 8 x 2 = 16 wells
- in total these are 40 wells, prepare a mastermix for 50 wells

<table>
<thead>
<tr>
<th></th>
<th>Total glutathione (GSH)</th>
<th>Oxidized glutathione (GSSG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mastermix for 50 wells</td>
<td>5000 µl</td>
<td>5500 µl</td>
</tr>
<tr>
<td>200mM NaH₂PO₄ - 10mM EDTA (pH 7.5)</td>
<td>5000 µl</td>
<td>5500 µl</td>
</tr>
<tr>
<td>dH₂O</td>
<td>3000 µl</td>
<td>2750 µl</td>
</tr>
<tr>
<td>10mM NADPH</td>
<td>500 µl</td>
<td>550 µl</td>
</tr>
<tr>
<td>12mM DTNB in DMSO</td>
<td>500 µl</td>
<td>550 µl</td>
</tr>
</tbody>
</table>

- Vortex this mastermix and store on ice
- Pipet samples (3x) and standards (2x) in the wells:
  - 10 µl for total glutathione (not incubated) (GSH standard)
  - 20 µl for oxidized glutathione (incubated) (GSSG standard)
- Add 500 µl GR stock (10 µl per sample) to the mastermix and vortex
- Add the mastermix to each well using the automatic multichannel
- 190 µl for total glutathione (GSH standard)
- 180 µl for oxidized glutathione (GSSG standard)
- Put the plate in the reader and start the measurement: M_GLUTATHIONE KINE
- after 20 sec shaking, DTNB reduction is measured during 5 min at 412 nm

Platereader protocols

**M_ASCORBATE ENDPOINT Protocol:**
Temperature: Setpoint 25°C | Shake: High for 2x 0:10 | Read: (A) 265 | 1 read point per well

**M_ASCORBATE KINE+AO Protocol:**
endpoint kinetics (total)
Temperature: Setpoint 25°C | Shake: High for 2x 0:10 | Read: (A) 265 | 1 read point per well

  - number of flashes per well per cycle: 20
  - kinetic time interval for 4 samples: 11s
  - number of cycles for 4 samples: 52 cycles
  - measuring time: 10m02s
  - kinetic time interval for 8 samples: 18s
  - number of cycles for 8 samples: 32 cycles
  - measuring time: 10m06s

**M_GLUTATHIONE KINE Protocol:**
kinecits (total)
Temperature: Setpoint 25°C | Shake: High for 3x 0:10 | Read: (A) 412 | 1 read point per well

  - number of flashes per well per cycle: 20
  - kinetic time interval for 4 samples: 20s
number of cycles for 4 samples: 14 cycles  
measuring time: 5m10s  
kinetic time interval for 8 samples: 22s  
number of cycles for 8 samples: 13 cycles  
measuring time: 5m16s

Data analysis

**ASCORBATE:**

Use the law of Lambert-Beer: $\Delta A = \varepsilon \cdot C \cdot d$

$\Delta A = A_{265 \text{ before addition of AO}} - A_{265 \text{ after 5 min addition of AO}}$

d = 0.5 cm

$\varepsilon_{265} = 14,000 \text{ M}^{-1} \text{cm}^{-1} = 0.014 \mu\text{M}^{-1} \text{cm}^{-1} = 0.014 \text{ (}\mu\text{mol})^{-1} \text{ cm}^{-1}$

$C = \frac{\Delta A}{\varepsilon \cdot d} = \frac{1}{0.014} \frac{\mu\text{mol cm}}{\text{L}} = \frac{1}{0.014} \frac{1}{0.5} \frac{1}{\text{L}}$

↓(200 µl well instead of 1 liter = 1/5000 dilution)

$C = \frac{\Delta A}{\varepsilon \cdot d} \cdot \frac{1}{0.014} \frac{1}{0.5} \frac{1}{200 \mu\text{l well}}$

Don’t forget to take the dilutions into account:

$C = \frac{x}{200 \mu\text{l well}} = \frac{x}{40 \mu\text{l extract}}$

↓(volume after neutralization to correct pH > volume before neutralisation)

$C = \frac{x}{40 \mu\text{l extract}} \cdot \frac{V_{after pH}}{V_{before pH}}$

↓(volume extraction buffer > 40 µl volume extract)

$C = \frac{x}{40 \mu\text{l extract}} \cdot \frac{V_{after pH}}{V_{before pH}} \cdot \frac{V_{extraction buffer}}{V_{before pH}}$

↓(total incubation volume > volume incubated extract and again neutralisation) → only for total AsA

$C = \frac{x}{40 \mu\text{l extract}} \cdot \frac{V_{after pH}}{V_{before pH}} \cdot \frac{V_{extraction buffer}}{V_{before pH}} \cdot \frac{V_{incubation with DTT}}{V_{incubated extract}} \cdot \frac{V_{before 2nd pH}}{V_{before 2nd pH}}$

↓(calculate per g fresh weight)

$C = \frac{x}{40 \mu\text{l extract}} \cdot \frac{V_{after pH}}{V_{before pH}} \cdot \frac{V_{extraction buffer}}{V_{before pH}} \cdot \frac{V_{incubation with DTT}}{V_{incubated extract}} \cdot \frac{V_{before 2nd pH}}{V_{before 2nd pH}} \cdot \frac{1000}{a \text{ mg}} \rightarrow \text{expressed in nmol/mg FW}$

$= \mu\text{mol/g FW}$

Example:

$C = \frac{x}{40 \mu\text{l extract}} \cdot \frac{V_{after pH}}{300 \mu\text{l}} \cdot \frac{250 \mu\text{l}}{100 \mu\text{l}} \cdot \frac{V_{after 2nd pH}}{250 \mu\text{l}} \cdot \frac{1000}{a \text{ mg}}$

References:

<table>
<thead>
<tr>
<th></th>
<th>Only reduced AsA</th>
<th>Total AsA</th>
<th>Only oxidized DHA</th>
<th>% reduced AsA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaves</td>
<td>1.5 µmol/g FW</td>
<td>2.5 µmol/g FW</td>
<td>1 µmol/g FW</td>
<td>60 – 105 %</td>
</tr>
<tr>
<td>Roots</td>
<td>0.15 µmol/g FW</td>
<td>0.5 µmol/g FW</td>
<td>0.35 µmol/g FW</td>
<td>60 – 105 %</td>
</tr>
</tbody>
</table>
GLUTATHIONE

Select a range that contains the linear phase of the highest standard (saturation can occur because limited products in the reaction) → normally you take from 0 to ca. 2.5 min

Calculate the slope in minutes, compare with the standard curve

Don’t forget to take the dilutions into account:

\[
C = x \frac{\text{pmol}}{200 \mu\text{l well}} = x \frac{\text{pmol}}{10 \text{ or } 20 \mu\text{l extract}}
\]

↓(volume after neutralization > volume before neutralisation)

\[
C = x \frac{\text{pmol}}{10 \text{ or } 20 \mu\text{l extract}} \cdot \frac{V_{\text{after pH}}}{V_{\text{before pH}}}
\]

↓(volume extraction buffer > volume extract)

\[
C = x \frac{\text{pmol}}{10 \text{ or } 20 \mu\text{l extract}} \cdot \frac{V_{\text{after pH}}}{V_{\text{before pH}}} \cdot V_{\text{extraction buffer}}
\]

↓(calculate per g fresh weight)

\[
C = x \frac{\text{pmol}}{10 \text{ or } 20 \mu\text{l extract}} \cdot \frac{V_{\text{after pH}}}{V_{\text{before pH}}} \cdot V_{\text{extraction buffer}} \cdot \frac{1000}{\text{mg}}
\]

→ expressed in pmol/mg

FW = nmol/g FW

Example:

\[
C = x \frac{\text{nmol}}{10 \text{ or } 20 \mu\text{l extract}} \cdot \frac{V_{\text{after pH}}}{300 \mu\text{l}} \cdot \frac{500 \mu\text{l}}{a \text{ mg}} \cdot \frac{1000}{a \text{ mg}}
\]

References:

<table>
<thead>
<tr>
<th></th>
<th>Total GSH</th>
<th>Only oxidized GSSG</th>
<th>Only reduced GSH</th>
<th>% reduced GSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaves</td>
<td>150 nmol/g FW</td>
<td>5 nmol/g FW</td>
<td>140 nmol/g FW</td>
<td>90 – 95 %</td>
</tr>
<tr>
<td>Roots</td>
<td>200 nmol/g FW</td>
<td>3 nmol/g FW</td>
<td>220 nmol/g FW</td>
<td>90 – 95 %</td>
</tr>
</tbody>
</table>
Principle: spectrophotometrics

Ascorbate assay: measuring decrease in reduced ascorbate at 265 nm
- Non-treated samples: measure only reduced AsA
  - Add AO (ascorbate oxidase)
  - AsA is specifically oxidized to non-absorbing DHA (dehydroascorbate)
- Treated samples: measure total ascorbate
  - Add DTT and its thiols will fully reduce the ascorbate pool to reduced AsA
  - Add AO
  - AsA is specifically oxidized to non-absorbing DHA

Glutathione assay: measuring increase in reduced DTNB at 412 nm
- Non-treated samples: measure total glutathione
  - Add GR (glutathione reductase)
  - Oxidized GSSG is specifically reduced to GSH
  - GSH reduces DTNB to an absorbing molecule
- Treated samples: measure only oxidised GSSG
  - Add 2-VP in order to complexate the GSH that is present in the sample
  - Centrifugate the sample twice in order to remove the 2-VP
  - Add GR
  - Oxidized GSSG is specifically reduced to GSH
  - Only not-complexated GSH reduces DTNB to an absorbing molecule