

# Lange Laboratory Data Acquisition and Processing

**NSF 2010 Project #0520140**

**Metabolomics: A functional genomics tool for deciphering functions of *Arabidopsis* genes in the context of metabolic and regulatory networks**

## **Method 1: Phytosterols and Tocopherols (PS1 protocol)**

Plant material (roughly 100 mg) was homogenized (Ball Mill MM301, Retsch, Haan, Germany) in the presence of liquid nitrogen and analytes were extracted in 4 mL CHCl<sub>3</sub>/MeOH (2:1, vol:vol; containing 1.25 mg/L epi-cholesterol as an internal standard) at 75°C for 60 min.

Samples were kept at room temperature for at least 1 h, solvents were evaporated to dryness (EZ2-Bio, GeneVac, Ipswich, UK), and the remaining residue was saponified in 2 mL 6 % KOH in MeOH (w / v) at 90°C for 60 min.

## **Method 1: Phytosterols and Tocopherols (PS1 protocol),**

### **continued**

After the mixture cooled down to room temperature, 1 mL n-hexanes and 1 mL H<sub>2</sub>O were added, the mixture was shaken vigorously for 20 sec, and the vials were centrifuged (3000 x g for 2 min) to achieve phase separation.

The hexane phase was transferred to a 2 mL glass vial, the saponification mixture extracted again with 1 mL n-hexanes as above, centrifuged as above, and the hexane phase added to the 2 mL glass vial containing the hexane phase from the first extraction.

The combined hexane phases were evaporated to dryness using a gentle stream of nitrogen, 50 µL of N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) were added to the residue, the sample was shaken vigorously for 20 sec, and the mixture transferred to a 2 mL autosampler glass vial with a 100 µL conical glass insert. After capping the vial, the reaction mixture was incubated at room temperature for at least 5 min.

## **Method 1: Phytosterols and Tocopherols (PS1 protocol),**

**continued**

GC/MS analyses were performed on an Agilent 6890N GC coupled to an Agilent 5973 inert MSD detector. Samples were loaded (injection volume 1  $\mu$ L) with a LEAP CombiPAL onto a HP-5MS fused silica column (30 m x 250  $\mu$ m; 0.25  $\mu$ m film thickness).

The temperatures of the injector and MSD interface were both set to 280°C. Analytes were separated at a flow rate of 1 mL/min using He as carrier gas and using a thermal gradient starting at 170°C (hold for 1.5 min), ramping first to 280°C at 37°C/min and then to 300°C at 1.5°C (hold for 5.0 min).

Eluents were fragmented in electron impact mode with an ionization voltage of 70 eV. Data were acquired using MSD ChemStation (Revision D.01.02.SP1) software. Background was subtracted and peaks were deconvoluted using AMDIS (Automatic Mass Spectral Deconvolution and Identification Software).

Analytes were identified based on their mass fragmentation patterns by comparison with those of authentic standards using the NIST (National Institute of Standards and Technology) Mass Spectral Search Program (Version D.05.00).

## **Method 1: Phytosterols and Tocopherols (PS1 protocol),**

### **continued**

Peak areas were obtained from the Total Ion Chromatogram (TIC) for all detectable peaks with a phytosterol mass fragmentation signature, whereas for the quantification of tocopherols (note that only  $\alpha$ -tocopherol accumulated to quantifiable levels in present set of samples) the Extracted Ion Chromatogram (EIC) at m/z 502 (detecting the trimethylsilyl derivative) was used.

Raw data were exported to Microsoft Excel and peak areas normalized to tissue mass and internal standard using Microsoft Access.

If the signal-to-noise ratio for a peak was equal to or lower than 3, a peak area of "0" was assigned.

To ensure low background signals a blank injection (followed by a shortened thermal gradient) was performed after each sample run.

Prior to sample analyses, and then after every 20 samples, a standard mix was run to evaluate the reproducibility of the analyses.

## **Method 2: Chlorophylls and Carotenoids (ISO1 protocol)**

Plant material (roughly 100 mg) was extracted three times with each 2 mL CHCl<sub>3</sub>/MeOH (2:1, vol:vol; containing 2.0 mg / L ubiquinone-10 as an internal standard) at room temperature for 15 min in the dark.

The organic extracts were combined in glass vials wrapped with aluminum foil to avoid light exposure, extracted against 1.5 mL 50 mM Tris / HCl (pH 7.5) / 1 M NaCl, and the vials were centrifuged (3000 x g for 2 min) to achieve phase separation.

The (lower) organic phase was transferred to a new tube in the dark, solvents were evaporated to dryness, analytes dissolved in 200 µL ethyl acetate, the mixture was filtered through a 0.45 µm nylon syringe filter and was transferred to a brown 2 mL autosampler glass vial with a 100 µL conical glass insert.

## **Method 2: Chlorophylls and Carotenoids (ISO1 protocol),**

**continued**

HPLC/DAD/MS analyses were performed using an Agilent Series 1100 HPLC system (including a G1315B diode array detector) coupled to an Agilent G2445D LC/MSD Trap SL mass spectrometer.

Samples were loaded (injection volume 20  $\mu$ L) onto a ProntoSil C30 column (250 x 4.6 mm; 5  $\mu$ m particle size; Bischoff Chromatography distributed via MAC-MOD Analytical, Chadds Ford, PA) equipped with a guard column of the same stationary phase material. The mobile phase consisted of (A) methanol, (B) H<sub>2</sub>O/MeOH (20:80; v:v) containing 0.2 % ammonium formate, and (C) tert-butyl methyl ether and gradient elution used the following gradient at a flow rate of 1 mL/min (modified from Fraser et al., 2000): 11 min at 95 % A / 5 % B (isocratically), a step change to 90 % A / 5 % B / 5 % C, a linear gradient (15 min) to 30 % A / 5 % B / 65 % C (hold for 5 min), followed by a conditioning phase to return to the initial conditions.

UV/VIS absorbance was monitored at 275, 287, 460 and 655 nm, and spectra were recorded from 250 to 750 nm in 2 nm increments. Analytes were identified and quantified by comparison of retention times and UV/VIS spectra with those of corresponding reference standards.

## **Method 2: Chlorophylls and Carotenoids (ISO1 protocol),**

### **continued**

To ensure proper peak assignments, eluting analytes from random samples were also ionized using atmospheric pressure chemical ionization and mass traces were acquired by single-ion monitoring in positive ion mode to detect the following mass ion transitions:  $m/z$  864  $\rightarrow$  680 (ubiquinone-10; internal standard),  $m/z$  538  $\rightarrow$  444 ( $\alpha$ - and  $\beta$ -carotene),  $m/z$  569  $\rightarrow$  551 (lutein), 894  $\rightarrow$  615 (chlorophyll *a*),  $m/z$  907  $\rightarrow$  629 (chlorophyll *b*),  $m/z$  872  $\rightarrow$  593 (pheophytin *a*).

The probe voltage was set to 4.0 kV, the capillary voltage was at 2200 V, the gas temperature was 350°C, and the nebulizer gas flow was 9.0 L/min.

Raw data were exported to Microsoft Excel and peak areas normalized to tissue mass and internal standard using Microsoft Access. If the signal-to-noise ratio for a peak was equal to or lower than 3, a peak area of “0” was assigned.

To ensure low background signals a blank injection was performed after every 10 samples.

Prior to sample analyses, and then after every 20 samples, a standard mix was run to evaluate the reproducibility of the analyses.

## Result highlights (environmental variables)

Sample number Sample name	02-058-01 NW-1	02-065-01 NW-2	02-072-01 NW-3	02-059-01 DLW-1	02-066-01 DLW-2	02-073-01 DLW-3	02-062-01 ILW-1	02-069-01 ILW-2	02-076-01 ILW-3
<b><u>Sterol</u></b>									
alpha Tocopherol	6	6	3	11	5	7	10	1	0
Cholesterol	331	417	547	422	358	422	313	271	304
Unknown #1*	469	556	420	598	348	436	<u>621</u>	<u>912</u>	<u>680</u>
Unknown #2*	419	293	392	366	340	266	311	361	621
Campesterol	4797	5543	6052	5668	4650	4719	4564	4163	5775
Stigmasterol	143	118	60	78	79	115	143	173	143
Sitosterol	19729	23777	21874	23001	19831	17345	19650	18312	19873
Isofucosterol	780	890	1228	<u>618</u>	<u>438</u>	<u>439</u>	881	493	997
Cycloartenol	1736	1969	1696	<u>813</u>	<u>552</u>	<u>437</u>	<u>3657</u>	<u>2165</u>	<u>3539</u>

**Follow-up experiment:**

**Expose Arabidopsis seedlings to varying light intensities**

