SOLID PHASE EXTRACTION:

ABOUT SPE: The SPE process can vary considerably with different needs, objectives and available equipment. Equipment such as columns, reagents, vacuum, extraction manifold and alternatives for each contribute considerably to this variation. The SPE protocol as written below is intended for use with the SPE Reagents and Materials list located in this section. This protocol may be amended to accommodate the specific needs of the testing lab, but should be done so at their own risk and discretion.

This procedure is intended for use with 1 mL of sample plasma, serum or tissue homogenate sample. This sample volume represents the minimum recommended volume for preparation in this assay. If attempting to prepare more or less than the 1 mL of sample mentioned, the proportion of materials and reagents should be adjusted accordingly. Things to consider when establishing the sample volume to be extracted and the final reconstitution volume are the anticipated concentration of isoprostane, the number of replicates to be performed in the assay, the anticipated IC_{50} value and the stringency of the experiment. Customarily, the stringency for this assay is at the 20% and 80% B/B_0. For higher stringency, adjust the $\%B/B_0$ acceptance thresholds to favor the 50% B/B_0 value.

NOTE: It is necessary to determine and adjust for incomplete recovery of isoprostane from the extraction columns. This can be achieved by the addition of a known quantity of 15-isoprostane F_{2t} standard (e.g. 5 ng) to an aliquot of one unknown prior to extraction, then analyzing both the spiked and unspiked samples. Upon analysis, calculate the difference to determine the percent recovery.

SPE MATERIALS AND REAGENTS

1. Magnesium Chloride ($MgCl_2$) (Sigma; M-9272)
2. Butylated Hydroxytoluene (BHT) (Sigma; B1378)
3. Methanol (MeOH) (Alfa Aesar; 32435)
4. Chloroform ($CHCl_3$) (Sigma; C-2432)
5. Triphenylphosphine (TPP) (Sigma; T84409)
6. Potassium Hydroxide (KOH) (Sigma; P-1767)
7. Hydrochloric Acid (HCl) (Fisher; A481-212)
8. Ethyl Acetate (Aldrich; 27,098-9)
9. Heptane (Sigma; H9629)
10. Evaporation Apparatus Suitable for 50 mL conical tubes and 20 mL reagent volumes
11. Water Bath suitable for 37°C
12. Analytical grade Nitrogen gas for evaporation of samples (see your local gas supply company)
13. Silica Sep Pak (Waters; WAT043400)
14. C18 Sep Pak (Waters; WAT043395)
15. 20 Position Extraction Manifold (Waters; WAT200609)
16. Vacuum source (Waters 110V, 60 Hz Vacuum pump; WAT085114 or equivalent)

SPE REAGENT PREPARATION

1. 0.043% $MgCl_2$ (store on ice prior to use)
2. MeOH + 0.05% BHT (w/v)
3. Folch Solution; Chlorform:Methanol (2:1) + 0.05% BHT (w/v) + 0.05% TPP (w/v) (store on ice prior to use) (BHT is an antioxidant)
4. Ethyl Acetate:Heptane (1:1)
5. Ethyl Acetate:MeOH (1:1)
6. pH 3 Deionized Water (pH with HCl and NaOH)
7. 15% KOH (w/v)

FREEING ESTERIFIED ISOPROSTANES

Only free isoprostanes are detected with this assay. Isoprostanes may be found adjoined to other molecules by an ester bond. For these esterified isoprostanes to be reflected in the assayed values they must have the ester bond removed. The following procedure will hydrolize the ester bond and allow for the analysis of total isoprostane. Alternatively, if only the

free isprostane at the time of collection is of interest, then skip this step and go directly to the Solid Phase Extraction Procedure later in this section.

1. Add 20 mL of ice-cold Folch Solution to a 50 mL conical tube followed by 1 mL of sample or tissue homogenate and vortex on high for 1 minute. Please note that the Folch Solution has a very low surface tension and may leak out of a poorly sealed tube during mixing.
2. Add 10 mL of ice-cold 0.043% $MgCl_2$ directly to the 50 mL conical tube and vortex on high for 1 minute.
3. Centrifuge for 3 minutes at 2500 x g to separate the phases of this mixture.
4. There will now be three phases (upper, white stuff, lower). Remove the upper layer by aspiration and discard. Poke through the remaining middle (white) layer with a pipette and carefully transfer the lower organic layer to a separate 50 mL conical test tube.
5. Evaporate the lower organic layer in the 50 mL conical tube under a stream of N_2. The dried sample will appear as an oily residue at the bottom of the vial.
6. Once dried, add 1 mL of MeOH + 0.05% BHT solution directly to sample and swirl by hand for 30 seconds to ensure the sample is adequately dissolved.
7. Add 2 mL of 15% KOH and swirl mixture for 30 seconds. (frees esterified isops.)
8. Incubate this mixture at 37°C for 30 minutes.
9. After incubation, add 17 mL of pH 3 water directly to 50 mL conical tube. Your sample is now ready for SPE.

SOLID PHASE EXTRACTION

The following procedure is performed under a constant vacuum. A negative pressure of ~5 psi is an appropriate benchmark but may require deviation for optimal flow rates and results.

1. The sample should be at a pH of 3 prior to SPE. Check and adjust the pH with 1 N HCl and 1 N NaOH accordingly.
2. Setup the extraction manifold and vacuum apparatus according to manufacturers instructions and affix the C_{18} Sep Pak column(s) to manifold with an appropriate waste container below each column.
3. Prewash the C_{18} Sep Pak column with 5 mL of EtOH followed by 5 mL of pH 3 Deionized Water.

NOTE: When running solutions through columns during washes, stop the solution when the solution meets the bed volume – do not allow the bed volume to run dry except when specified.

4. Load the sample to the column and flow through the column at a flow rate of 1 mL per minute. (~1 drip/sec)
5. Wash the column with 10 mL of pH 3 Deionized Water followed by 10 mL of Heptane.
6. Remove waste material collection device and insert sample collection tubes appropriate for the volume and solvent being used.
7. Elute the sample from the column with 10 mL Ethyl Acetate:Heptane until the column is dry.
8. Remove the eluted sample, add Sodium Sulfate to absorb aqueous layer, and set aside for the next phase in extraction. Affix the Silica Sep Pak column(s) to manifold with an appropriate waste container below each column.
9. Prewash the Silica Sep Pak column with 5 mL of Methanol followed by 5 mL of Ethyl Acetate.
10. Load the sample collected from the C_{18} Sep Pak to the column and flow through the column at a flow rate of 1 mL per minute.
11. Wash the column with 5 mL of Methanol followed by 5 mL of Ethyl Acetate.
12. Remove waste material collection device and insert sample collection tubes appropriate for the volume and solvent being used.
13. Elute the sample from the column with 5 mL Ethyl Acetate:Methanol until the column is dry
14. Evaporate the eluted sample under a stream of N_2. The dried sample will appear as an oily residue at the bottom of the vial.

NOTE: Storage of isoprostane samples is ideal in the concentrated lipid form as resulting from the drying procedure. Store at –80°C under inert gas until the time of assay.

15. Reconstitute the sample in a known amount of Dilution Buffer and proceed to the Assay Procedure.

If you know your starting volume, reconstitute the sample in 1/2 the volume to concentrate it, and then dilute it.