Supporting Information

"A Novel LC-ESI/MS/MS^n Method for the Characterization and Quantification of 2′-Deoxyguanosine Adducts of the Dietary Carcinogen 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine by 2-D Linear Quadrupole Ion Trap Mass Spectrometry"

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Supporting Information – Experimental Details and Discussion on the Ion Suppression Studies

Experimental Methods:

The samples were incubated by one of the various preparation protocols described below. Then, the solvents used for the sample treatments were transferred to a total recovery capillary LC vial and dried by vacuum centrifugation. The extracts were resuspended in 1:1 DMSO:H_2O containing a known amount of dG-C8-PhIP standard (5 pg) and assayed by LC-ESI/MS/MS^n. The degree of ion suppression was estimated by measurement of the signal relative to the response of the pure standard. Samples were assayed in triplicate.

Solvents. The solvents examined were HPLC-grade CH_3OH (Fisher, P/N A452-4), HPLC-grade CH_3CN (JT Baker, P/N 9017-03), HPLC-grade acetone (Aldrich, 650501), 200 proof C_2H_5OH (Pharmaco, P/N 11100200-CSPP, Brookfield, CT), reagent alcohol

(Fisher, P/N A995-4), high-purity B & J Brand® CH_3OH (Burdick and Jackson, P/N 230-4), and high purity B & J Brand® CH_3CN (Burdick -and Jackson, P/N 015-4). Each solvent (1 mL) was added to total recovery capillary LC vials with a glass Pasteur pipet and incubated in the vials for 5 min at room temperature, followed by vacuum centrifugation.

SPE cartridges (individual components). The SPE cartridges were disassembled into the 1-mL polypropylene cartridge reservoir, resin, and additionally-washed polyethylene frit components. The frits were placed into microcentrifuge tubes (1.5 mL) (Eppendorf) and the potential of the polyethylene frits to provoke both ion suppression and irreversible binding of dG-C8-PhIP was investigated. High-purity B & J Brand® CH_3OH (1 mL) was added to the microcentrifuge tubes containing the frits with a glass Pasteur pipet and incubated at room temperature for 5 min. Thereafter, the CH_3OH and any extracted contaminants from the frits were retrieved and evaporated to dryness by vacuum centrifugation in total recovery capillary LC vials. The concentrated extract was spiked with dG-C8-PhIP (5 pg). In the second experiment to assess binding of dG-C8-PhIP to the frits, the CH_3OH solvent used for extraction of the frit contained dG-C8-PhIP (5 pg). The response of the dG-C8-PhIP spiked after concentration of the CH_3OH extract represented ion suppression effects of the frits, while the sample spiked pre-extraction represented both the % recovery of adduct and ion suppression effects.

The resins (25 mg) were placed into separate microcentrifuge tubes and high-purity B & J Brand® CH_3OH (1 mL) was added with a glass Pasteur pipet. After incubation (5 min) at room temperature, the sample was centrifuged (10,000*g* for 1 min), and the

supernatant was carefully transferred to a total recovery capillary LC vial. This process was repeated twice more, for a total of three CH_3OH washes of the resin.

The empty polypropylene cartridge holders were placed into an SPE manifold with the ports closed. High-purity B & J Brand® CH_3OH was added to the cartridge with a glass Pasteur pipet and incubated 5 min at room temperature, followed by transfer into total recovery capillary LC vials, and evaporation to dryness.

Results and Discussion:

During the course of SPE method development for adduct enrichment prior to LC-ESI/MS/MS^n analysis, we observed a >95% decrease in the signal intensity of the dG-C8-PhIP adduct spiked in DNA digest and purified by SPE. We observed that much of the suppression in the signal of dG-C8-PhIP still arose when the adduct was processed through a mock enzyme digest (absence of enzymes and DNA), followed by SPE. This suggested that one or more components among the solvents, SPE cartridge, or other consumable items – rather than the biological matrix – were major contributors to the diminution of the signal. Therefore, all of the consumable items used for sample preparation were investigated as potential sources of contaminants that could lead to ion suppression of the dG-C8-PhIP signal.

A significant difference in the extent of ion suppression was observed between the HPLC-grade and high-purity B & J Brand® CH_3OH solvents (Supporting Information, Figure 1). Since HPLC-grade CH_3OH was initially used for the elution of the dG-C8-PhIP adduct from the SPE cartridge, some of the contaminants present in the HPLC-grade CH_3OH, which were recovered with the adduct eluted from the SPE cartridge,

could contribute to the ion suppression effects. Therefore, all of the subsequent experiments and results described used high-purity B & J Brand® organic solvents and water at every step of DNA adduct preparation, in an effort to reduce the ion suppression.

The individual components of the SPE Isolute C18(EC) cartridge (the C18 resin, the polypropylene reservoir, and the polyethylene frits) were also examined as potential sources of ion suppression (Supporting Information, Figure 2). The polyethylene frits were a major source of ion suppression, particularly in the second batch of SPE resin assayed; these disparate results are indicative of batch-to-batch variations that may have been responsible in part for variations in the degree of ion suppression or adduct recovery observed in experiments performed on differing days. Moreover, dG-C8-PhIP was found to strongly bind to the polyethylene frits, as shown by the fact that the signal from the extract of the adduct that had been co-incubated with the frits in CH_3OH was 60% lower than the signal from the extract with the adduct spiked into the CH_3OH extract after removal of the frits. These findings show that both ion suppression effects and incomplete recovery of the adduct contribute to the diminution of the dG-C8-PhIP signal. In an effort to replace the polyethylene frits used in the manufacture of most SPE cartridges, we examined Teflon and stainless steel frits, all with the same Isolute C18(EC) SPE system. The recovery of the dG-C8-PhIP standard was very poor when we used either the stainless steel frits or – surprisingly – the Teflon frits, presumably due to binding of the adduct to the frit. The use of high-purity B & J Brand® CH_3CN or HPLC-grade acetone as the eluent, instead of CH_3OH, did not increase the recovery of dG-C8-PhIP. Similar ion suppression effects were also observed with the use of SPE cartridges from other vendors.

Various brands of pipet tips, microcentrifuge tubes, and capillary LC vials were examined as possible sources of ion suppression; however, there was no apparent ion suppression provoked by any of these consumable items. Alternate SPE systems are currently under investigation, in efforts to further reduce ion suppression effects and to improve both adduct recoveries and assay sensitivity. At the current time, the response in signal of the dG-C8-PhIP or dG-C8-[$^2H_3$]-C8-PhIP adduct that is processed through the DNA digest (1–100 adducts per 10^8 bases) and the SPE procedure is ~30-50% relative to the pure standard.

Supporting Information – Figure Legends

Figure 1. Ion suppression of the dG-C8-PhIP standard provoked by various solvents of differing purities. (A) HPLC-grade organic solvents, (B) high-purity B & J Brand® solvents. *The response of dG-C8-PhIP assayed from concentrated HPLC-grade CH_3OH was significantly lower than the pure standard ($P < 0.01$)

Figure 2. Ion suppression of the dG-C8-PhIP standard provoked by the individual components of the Isolute C18(EC) SPE cartridges with additionally washed polyethylene frits. (A) First trial (from left to right): Standard (dG-C8-PhIP); CH_3OH extract of resin – 1st wash; CH_3OH extract of resin – 2nd wash; CH_3OH extract of resin – 3rd wash; polyethylene frit spiked with dG-C8-PhIP post-incubation and after removal of the frit and concentration of the CH_3OH extract; cartridge holder (incubation of the polypropylene cartridge holder with CH_3OH). (B) 2nd batch of SPE resin (from left to

right): Standard (dG-C8-PhIP); CH_3OH extract of resin – 1st wash; CH_3OH extract of resin – 2nd wash; CH_3OH extract of resin – 3rd wash; polyethylene frit co-incubated with dG-C8-PhIP; polyethylene frit spiked post incubation and after removal of the frit and concentration of the CH_3OH extract; cartridge holder (incubation of the polypropylene cartridge holder with CH_3OH. std (dG-C8-PhIP standard).

Figure 3. Representative calibration curve of dG-C8-[^2H_3C]PhIP at 5 adducts per 10^7 DNA bases, equivalent to 25 pg injected on column with unlabeled dG-C8-PhIP present at a level equivalent to 1 to 100 adducts per 10^8 DNA bases (for 27 μg of DNA).

Figure 4. Comparison of product ion spectra (MS^3) of dG-C8-PhIP (A) standard, and (B) from CT DNA (3 adducts per 10^8 DNA bases)