Chapter 2

Measuring the Activity of 1-Deoxy-D-Xylulose 5-Phosphate Synthase, the First Enzyme in the MEP Pathway, in Plant Extracts

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Abstract

The first enzyme in the methylerythritol phosphate (MEP) pathway is 1-deoxy-D-xylulose 5-phosphate (DXP) synthase (DXS). As such this enzyme is considered to be important in the control of plastidial isoprenoid production. Measuring the activity of DXS in plant extracts is therefore crucial to understanding the regulation of the MEP pathway. Due to the relatively low amounts of DXS, the activity of this enzyme can only be measured using highly sensitive analytical equipment. Here, a method is described to determine the DXS enzyme activity in a crude plant extract, by measuring DXP production directly using high performance liquid chromatography linked to a tandem triple quadrupole mass spectrometry detector (LC-MS/MS).

Key words DXS, Enzyme assay, Isoprenoid biosynthesis, LC-MS/MS

1 Introduction

All isoprenoids are produced from the same C5 isoprene units, isopentenyl diphosphate and dimethylallyl diphosphate. In the cytosol these isoprenoid building blocks are biosynthesized through the well-known mevalonate pathway, whereas these same precursors are biosynthesized in the plastids by the recently discovered 2-C-methyl-erythritol 4-phosphate (MEP) pathway [1, 2]. In this pathway, DXS condenses pyruvate and glyceraldehyde 3-phosphate to form 1-deoxy-D-xylulose 5-phosphate (DXP) in the first step towards the formation of plastidic isoprenoids. The MEP pathway provides the precursors for synthesizing products with diverse roles in plant energy metabolism, photosynthesis and plant–insect interactions. Additionally, the MEP pathway provides the precursors for the biosynthesis of many end products with

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considerable economic value, including the anticancer drugs paclitaxel, vincristine, and vinblastine, common flavor and fragrance compounds such as geraniol, linalool, and menthone, cosmetics such as shikonin, and other industrial raw materials such as the monoterpenoid olefinic hydrocarbons used to make turpentine. A detailed knowledge of the regulation of the early steps which provide common precursors for diverse downstream isoprenoid pathways may lead to increased production of commercially valuable isoprenoids. However, the processes by which this pathway is regulated are still poorly understood.

The amount of end products produced by a metabolic pathway depends on its flux, which in turn is dependent on the rate at which the individual enzymatic steps convert their respective intermediates. Identifying the enzymatic steps most important for metabolic flux will aid the elucidation of the regulatory mechanisms responsible for isoprenoid biosynthesis. To achieve this, it is crucial to measure the activities of the individual enzymes in plant extracts. As the first committed enzyme, DXS is considered to be an important enzyme in the regulation of the MEP pathway. Measurement of DXS activity was first accomplished using recombinant proteins following the initial isolation of their cDNAs from *E. coli* [3] and mint [4]. In the former case, ^{14}C-labelled pyruvate was spiked into the reaction mixture, and the radioactive products were separated by TLC, a technique adopted elsewhere for analyzing DXS activity [5]. In the latter case, the DXP product of mint DXS was derivatized for gas chromatographic analysis by removal of the 5-phosphate and trimethylsilylation of the free hydroxyls. The activity of DXS has also been confirmed on a qualitative level by bacterial complementation through the transformation of *E. coli* strains deficient in DXS activity [6–8].

Measuring the activity of DXS in plant tissue, however, is another matter. Pyruvate and glyceraldehyde 3-phosphate are intermediates of a multitude of other metabolic pathways, and their respective enzymes will also be present in plant protein extracts, competing with DXS for exogenous substrates. Furthermore, the concentration and activity of DXS are very low on a cellular scale, necessitating highly sensitive methods to detect its activity. To overcome these problems methods were developed to derivatize DXP with 3,5-diaminobenzoic acid and measure the fluorescence emitted by the quinaldine product [9]. Another approach uses isotope ratio mass spectrometry to detect the $^{13}CO_2$ emitted by the DXS reaction when using labelled pyruvate as substrate [10]. A further solution to low sensitivity was proposed involving the reductive amination of the DXP product with anthranilic acid. The fluorescent product is then separated by HPLC and detected by a fluorescence detector [11]. However, its sensitivity is limited to measuring recombinant proteins. Other methods overcome the problem of low enzyme activities in plant extracts by increasing the DXS enzyme concentrations by isolating plastids [12, 13],

methods that are themselves prone to high variability. Thus far, no published method of measuring DXS activity is capable of reproducibly quantifying the low levels of DXS in plant extracts.

Here we describe a fast and simple method to measure DXS activity. A crude plant protein extract is prepared which is added to an enzyme reaction mixture consisting of the glyceraldehyde 3-phosphate and pyruvate substrates as well as the thiamine diphosphate and Mg^{2+} cofactors. After the reaction is stopped, the DXP product is detected using the sensitivity provided by LC-MS/MS.

2 Materials

Use analytical grade reagents and ultrapure deionized water. Extra care should be taken that the water used for liquid chromatography triple quadrupole mass spectrometry (LC-MS/MS) analyses should be purified to a resistivity of 18 MΩ at 25 °C and the acetonitrile should be at least HPLC grade. All LC-MS/MS solvent additives used must be LC-MS grade. All reagents and plant material should be kept on ice during the enzyme extraction and enzyme assay preparation procedure (unless indicated otherwise). Waste disposal regulations must be meticulously followed.

2.1 Crude Enzyme Extraction

1. Stock extraction buffer: 50 mM Tris–HCl pH 8.0 (*see* **Note 1**), 10 % glycerol (*see* **Note 2**), 0.5 % Tween 20, 1 % polyvinylpyrrolidone (PVP) (average molecular weight 360,000), 2 mM imidazole, 1 mM NaF, and 1.15 mM molybdate (*see* **Note 3**). The stock extraction buffer is made to a volume of 100 mL and can be stored for 1 week at 4 °C.
2. Prepare fresh a small quantity of 1 M dithiothreitol (DTT), a small quantity of 1 M ascorbic acid and a small quantity of 100 mM thiamine pyrophosphate (TPP). These reagents can be prepared in a final volume of 1 mL or scaled up for larger numbers of extractions (*see* **Note 4**). Keep the solutions on ice.
3. Protease inhibitor cocktail for plant cell extracts (Sigma-Aldrich). Store at −20 °C (*see* **Note 5**).
4. Microcentrifuge tubes of 2 mL capacity.
5. Vertical rotator (Stuart rotator SB3, VWR, or equivalent).
6. Refrigerated microcentrifuge (Eppendorf centrifuge 5415R, or equivalent).

2.2 DXS Enzyme Assay

1. Assay buffer: 100 mM Tris–HCl, pH 8.0 (*see* **Note 1**), 20 % glycerol (*see* **Note 2**), 20 mM $MgCl_2$ (*see* **Note 6**). Store for up to a week at 4 °C.
2. Prepare fresh a small quantity of 1 M sodium pyruvate (*see* **Note 4**). Dissolve 110.04 mg in 1 mL water and keep on ice.

3. Freshly prepared 1 M DTT and 100 mM TPP (*see* Subheading 2.1, **item 2**).
4. DL-Glyceraldehyde 3-phosphate (GAP) supplied as a 45–55 mg/mL solution (Sigma-Aldrich). Store as aliquots at −20 °C.
5. Water bath set at 25 °C.
6. Chloroform. Store at room temperature in a solvent cabinet.

2.3 DXP Detection

1. 5 M ammonium acetate stock solution: Dissolve 19.3 g LC-MS grade ammonium acetate in 50 mL water and store at 4 °C.
2. Liquid chromatography (LC) solvents: 20 mM ammonium acetate, pH 10.0 (solvent A), 80 % acetonitrile containing 20 mM ammonium acetate, pH 10.0 (solvent B) (*see* **Note 7**).
3. 1 mg/mL DXP (Sigma-Aldrich) dissolved in 5 mM ammonium acetate, 50 % acetonitrile (*see* **Note 8**).
4. 1 mg/mL ^{13}C isotopically labelled DXP dissolved in water (*see* **Note 9**).
5. XBridge BEH Amide column (150 × 2.1 mm, 3.5 μm, Waters) with an XBridge BEH Amide Sentry guard cartridge (10 × 2.1 mm, 3.5 μm, Waters) and a high pressure pre-column filter (SSI High Pressure Pre-column Filter, Sigma) or equivalent.
6. Agilent 1200 HPLC system (Agilent Technologies) or equivalent.
7. API 3200 triple quadrupole mass spectrometer (Applied Biosystems) or equivalent.

3 Methods

All steps used for extraction of enzymes should be carried out on ice, unless otherwise specified. This method has been optimized for measuring DXS activity in leaf material of *Arabidopsis thaliana* and has also been tested on leaf material from *Populus tremuloides*. Measuring DXS activity in other plant species or tissues might require additional optimization.

3.1 Crude Enzyme Extraction

1. Prepare the extraction buffer by adding 100 μL of 1 M DTT, 100 μL of protease inhibitor cocktail, 10 μL of 1 M ascorbic acid and 10 μL of 100 mM TPP to a graduated cylinder and make up to 10 mL with the stock extraction buffer (*see* **Note 10**). Keep the extraction buffer on ice.
2. Homogenize plant material to a fine powder in liquid nitrogen in a pre-cooled mortar and pestle. Transfer the homogenized plant material to a plastic tube kept frozen in liquid nitrogen or on dry ice.

3. Weigh between 20 and 25 mg fresh weight plant material in a 2 mL microcentrifuge tube pre-cooled in liquid nitrogen. It is crucial that the plant material does not thaw; work quickly and return the weighed material to liquid nitrogen or dry ice (*see* **Note 11**). Note the exact weight.
4. Add 1 mL of extraction buffer to each microcentrifuge tube and mix gently on a vertical rotator at 20 rpm for 15 min at 4 °C (*see* **Note 12**). Transfer tubes to a pre-cooled microcentrifuge and centrifuge at 16,000 × *g* for 20 min at 4 °C. The supernatant can now be used in the enzyme assays.

3.2 DXS Enzyme Assay

1. Prepare the enzyme reaction mixture by adding 0.25 µL of 1 M DTT, 1 µL of 100 mM TPP, 1 µL of 1 M pyruvate, 1 µL of the phosphatase inhibitor cocktail (*see* **Note 3**), 1 µL of protease inhibitor cocktail, and 1 µmol of the GAP solution (*see* **Note 13**) to 50 µL assay buffer. Add enough water to reach a final volume of 70 µL (Table 1).
2. Add 30 µL of the enzyme extract to the reaction mixture and incubate in a 25 °C water bath for 2 h (*see* **Note 14**).
3. Stop the enzyme reaction by adding one volume of chloroform and vortexing vigorously (*see* **Note 15**). Centrifuge at maximum velocity in a microcentrifuge to achieve phase separation and

Table 1
Overview of the amounts of the different reagents necessary to prepare a single DXS enzyme reaction with a final volume of 100 µL

Reagent	Stock concentration	Assay concentration	Vol. added
Assay buffer	100 mM Tris–HCl 20 mM $MgCl_2$ 20 % Glycerol	50 mM Tris–HCl 10 mM $MgCl_2$ 10 % Glycerol	50 µL
DTT	1 M	2.5 mM	0.25 µL
TPP	100 mM	1 mM	1 µL
PhI[a]	200 mM imidazole 100 mM NaF 115 mM sodium molybdate	2 mM imidazole 1 mM NaF 1.15 mM sodium molybdate	1 µL
PrI[b]	Solution	1 %	1 µL
Sodium pyruvate	1 M	10 mM	1 µL
GAP	Solution	10 mM	3.4 µL [c]
Water			12.35 µL
Enzyme			30 µL

[a]Phosphatase inhibitors
[b]Protease inhibitor cocktail
[c]Volume of GAP used depends on the concentration of the solution purchased (*see* **Note 13**)

transfer 45 μL of the aqueous upper phase to a HPLC vial fitted with a 200 μL insert. Add 5 μL of a 10 ng/μL [3,4,5-$^{13}C_3$] DXP internal standard (*see* **Note 16**). Dilute this solution with one volume (50 μL) of methanol to approximate the initial mobile phase and improve peak shape.

3.3 DXP Detection with LC-MS/MS

1. Optimize the instrument parameters for detecting DXP by infusing a 100 μg/mL solution of DXP dissolved in 5 mM ammonium acetate in 50 % acetonitrile into the mass spectrometer (*see* **Note 17**) using the automatic optimization procedure under negative ionization mode. The optimized parameters will be different for different LC-MS/MS instruments, *see* Table 2 for the optimized parameters of an API 3200 LC-MS/MS.
2. Liquid chromatographic separation of DXP is achieved with a HILIC column (XBridge BEH Amide) using the following LC parameters: column temperature = 25 °C, injection volume = 20 μL, flow rate = 0.5 mL/min, total run time = 20 min. Use a solvent gradient program starting with a linear gradient from 0 to 20 % solvent A over 0.5 min, isocratic separation at 20 % A until 10 min, a linear increase to 30 % A by 11 min, hold at 30 % A until 15 min, return to initial conditions of 0 % A at 15.1 min and equilibrate at 0 % A until 20 min.
3. Ionization was achieved using eletcrospray ionization with a Turbospray ion source operating under negative ionization mode. The ion spray voltage was maintained at −4,500 eV and the turbo gas temperature at 700 °C. The nebulizing gas was set at 70 psi, the heating gas at 30 psi, the curtain gas at 30 psi and the collision gas at 10 psi. The ionization parameters also need to be optimized for different LC-MS/MS systems.

Table 2
Selected reaction monitoring transitions and conditions used in the API 3200 LC-MS/MS

Analyte	Precursor *m/z*	Product *m/z*	EP[a] (V)	CEP[b] (V)	CE[c] (V)	CXP[d] (V)
DXP	312.9	138.9	−8	−12	−18	−4
		78.9	−7	−28	−42	0
DXP-$^{13}C_3$	215.9	140.9	−8	−12	−18	−4
		78.9	−7	−28	−42	0

The dwell times used were 0.15 s with a declustering potential (DP) of −20 V. Both Q1 and Q3 quadrupoles were operated at unit resolution
[a]Entrance potential
[b]Cell entrance potential
[c]Collision energy
[d]Cell exit potential

4. The optimized parameters of the triple quadrupole mass spectrometer are shown in Table 2. DXP is detected as a mass-to-charge ratio (m/z) of 138.9, the product ion of the DXP [M − 1] precursor ion (m/z 212.9). The labelled internal standard is detected as precursor ion → quantifier ion: m/z 215.9 → 140.9 (*see* Fig. 1). The identities of DXP and its internal standard can be verified using the precursor ion → qualifier ion combinations m/z 212.9 → 78.9 and m/z 215.9 → 78.9, respectively (*see* **Note 18**). The product ion and qualifier ion obtained after fragmentation are shown in Fig. 1.
5. Construct a calibration curve with DXP in the range of 0.1–10 ng/μL.

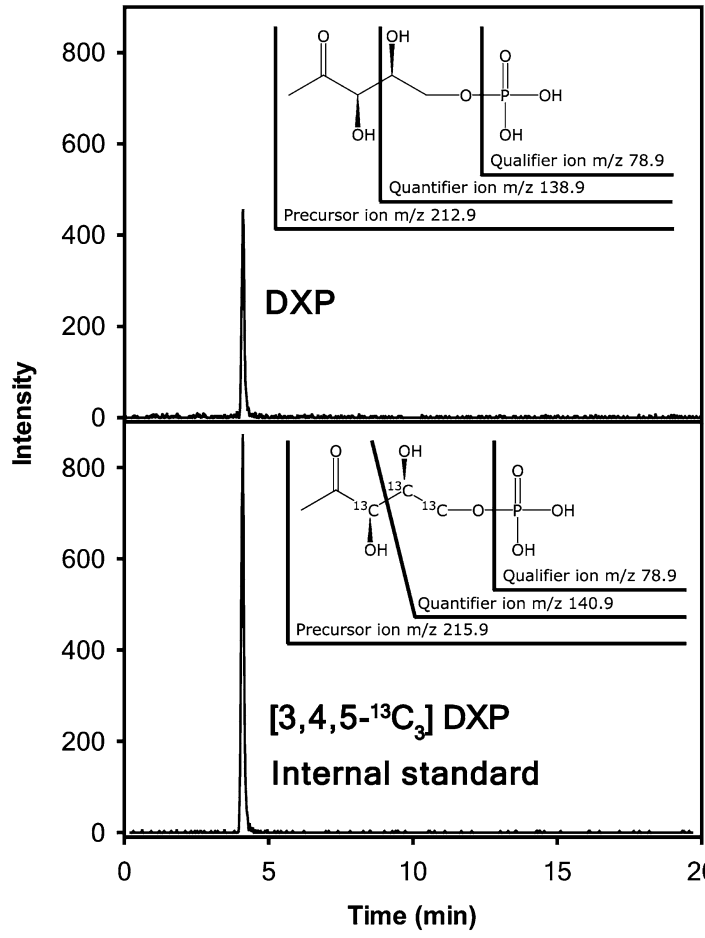

Fig. 1 Representative chromatogram for the DXS assay product, DXP, and the internal standard, [3,4,5-$^{13}C_3$] DXP. The fragmentation of DXP and the internal standard to produce the quantifier ions m/z 138.9 and m/z 140.9, as well as the qualifier ion m/z 78.9, is also shown

6. Analyst 1.5 software (Applied Biosystems) was used for data acquisition and processing. Quantify the DXP produced by the DXS enzyme reaction using the calibration curve and normalize it to the detected internal standard to correct for any ion suppression effects (*see* **Note 19**). Lastly adjust the DXP amounts for the dilution due to the added acetonitrile and internal standard (*see* **Note 20**).

4 Notes

1. Prepare a 1 M Tris–HCl pH 8.0 buffer that can be stored at room temperature and later diluted to the desired concentration when preparing buffers. We usually make a 1 L solution by dissolving 121.1 g Tris in 600 mL water in a graduated cylinder or glass beaker. Adjust the pH by adding HCl, starting with concentrated acid at first and switching to more dilute acid (e.g., 1 N) when nearing the desired pH. When diluting concentrated acids it is important to remember to add the acid to the water. After the buffer was adjusted to pH 8.0, bring the final volume to 1 L.
2. Glycerol is stored as a 50 % solution. This makes it much easier to handle than the viscous undiluted reagent.
3. The phosphatase inhibitor cocktail consisting of imidazole, NaF and molybdate are prepared as a 100× stock solution and stored at 4 °C. Dissolve 1.36 g imidazole, 0.42 g NaF, and 2.78 g molybdate in 100 mL water. Use protective clothing, gloves and a face mask when weighing NaF. Due to its toxicity, protective clothing and gloves should be used when handling any of the reagents containing NaF.
4. All the reagents that are more labile are prepared fresh and added to the extraction buffer shortly before use. To save on consumables and expenses, small quantities are prepared by weighing an approximate amount in 2 mL eppendorf tubes and then adding the appropriate amount of water to obtain the desired concentration. This approach does not take into consideration the volume of the reagent in the final solution, but the slight decrease in reagent concentration so achieved will have no effect on the final enzyme reaction. For example, to make a 1 M solution of DTT of about 1 mL, weigh between 100 and 200 mg in a 2 mL eppendorf tube and write down the exact weight. Now divide the weighed amount of DTT by 154.2 and multiply by 1,000 to get the volume of water (in µL) to add to the 2 mL eppendorf tube to get a 1 M solution. In other words, dissolve 154.2 mg DTT in 1 mL to prepare a 1 M solution, dissolve 176.12 mg L-ascorbic acid in 1 mL to prepare a 1 M solution, and dissolve 46.08 mg TPP in 1 mL to prepare a 100 mM solution.

5. The protease inhibitor cocktail is supplied as a solution in dimethyl sulfoxide (DMSO) which solidifies when kept on ice. Thaw prepared aliquots at room temperature and return to −20 °C as soon as possible to minimize degradation.
6. Prepare a 1 M $MgCl_2$ solution to dilute into the assay buffer to achieve the desired concentration. Weigh out 9.5 g anhydrous $MgCl_2$, dissolve in 100 mL water and store at room temperature.
7. Ammonium acetate is highly hygroscopic and should be kept under argon. To ease preparation of the solvents used for LC a stock solution of 5 M is prepared and stored at 4 °C. This is then diluted to obtain a 10× concentration and adjusted to the correct pH using LC-MS grade ammonium hydroxide. Add 4 mL of the 5 M ammonium acetate stock solution to approximately 80 mL water and add concentrated ammonium hydroxide to reach pH 10. Finally adjust the volume to 100 mL. The ammonium acetate buffer is then diluted with either water or acetonitrile to prepare the respective LC solvents. Add acetonitrile to the aqueous ammonium acetate solution for easy mixing of the salt with the organic solvent.
8. Prepare a 10 mM ammonium acetate solution by diluting the 5 M ammonium acetate stock by adding 200 μL of the stock to a graduated cylinder and make up to 100 mL with water. Then add 1 volume 10 mM ammonium acetate to 1 volume acetonitrile.
9. ^{13}C isotopically labelled DXP is not commercially available, but could be prepared enzymatically as described [14]. The simplest method is to use recombinant *Escherichia coli* DXS to produce [1,2-$^{13}C_2$] DXP by using [2,3-$^{13}C_2$] pyruvate as substrate.
10. Adjust the volumes according to how many extractions are planned, allowing 1 mL of extraction buffer per extract.
11. When the enzyme activity is normalized to protein content, it is not necessary to determine the exact weight, and only an approximate amount of plant material need to be transferred to a pre-cooled microcentrifuge tube. Another alternative, which works well for *Arabidopsis thaliana*, is to freeze dry the plant material and then weigh 5 mg of the dried material in a microcentrifuge tube at room temperature. The dried material should, however, still be kept on ice and be stored at -20 °C before weighing.
12. When adding the extraction buffer to the microcentrifuge tubes pre-cooled in liquid nitrogen, the extraction buffer will freeze on the tube surface, hindering proper mixing of the plant material with the extraction buffer. To circumvent this problem, put the tubes on ice for exactly 2 min before adding the extraction buffer. This allows the microcentrifuge tubes to

warm sufficiently to minimize frozen extraction buffer but still keep the plant material in a frozen state.

13. Use the following equation to calculate the amount of GAP to use in each 100 µl enzyme assay to obtain a 10 mM concentration: µL GAP to be used = 170.06/(concentration of GAP reagent in units of mg/mL). For example, use 170.06/50 = 3.4 µL GAP for a 50 mg/mL solution.
14. Although the maximum temperature for the *A. thaliana* DXS assay is at approximately 40 °C, the technical variation between enzyme assays is lower at room temperature.
15. The chloroform extraction is actually done to remove hydrophobic compounds from the reaction mixture, which otherwise interfere with the separation of the DXP on a HILIC column. The chloroform extraction was consequently also found to be a sufficient procedure for stopping the DXS enzyme reaction.
16. Although LC-MS/MS is a very sensitive analytical technique, it suffers from the disadvantage of ion-suppression. For a compound to be detected with a mass spectrometer it needs to contain a charge. In the case of LC-MS/MS, the compound is ionized through electro spray ionization in the ion source by applying electrical charge to the LC eluent. The presence of other molecules in the eluent, competing with the compound of interest for the available charge, will influence the ionization efficiency of the compound, and hence its detection by the mass spectrometer. It is thus usually not possible to measure the absolute quantity of a compound in a complex mixture, such as an enzyme reaction of a crude plant extract, when using an external standard curve to calculate the amount of the analyte. To compensate for the ion-suppression occurring, a known amount of ^{13}C isotopically labelled DXP is added to the enzyme reaction product. The labelled DXP will have the same ionization efficiency of the unlabelled DXP, but can be distinguished by its mass difference. The labelled DXP can thus be used as internal standard to quantify the absolute amount of DXP. However, when no internal standard is used, it will still be possible to measure the relative DXS activities of different plant extracts, as long as the plant tissues used have a similar matrix composition.
17. Dissolving DXP in 50 % acetonitrile containing 5 mM ammonium acetate significantly increases the ionization efficiency and also more closely represents the ionization conditions during a LC-MS/MS run.
18. Although the qualifier ion (m/z 78.9) gives a more sensitive signal, the less sensitive quantifier ion (m/z 138.9, and m/z 140.9 for the internal standard) is used for quantification to minimize potential background signals.

19. Use the following equation to normalize the detected DXP relative to the internal standard: DXP = ((DXP measured) × (Internal standard added))/(Internal standard measured). For example, if 1 ng internal standard was injected and 0.55 ng DXP and 0.75 ng internal standard was detected, the absolute amount of DXP injected will be $(0.55 \times 1)/0.75 = 0.73$ ng.
20. To account for the dilution due to the addition of acetonitrile and the internal standard, multiply the amount of DXP detected by 2 and divides this by 0.9. Using the example of **Note 19**, this means that the undiluted amount of DXP will be $(0.73 \times 2)/0.9 = 1.62$ ng/μL if 1 μL was injected.

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