CHAPTER TEN

Quantifying the Metabolites of the Methylerythritol 4-Phosphate (MEP) Pathway in Plants and Bacteria by Liquid Chromatography–Triple Quadrupole Mass Spectrometry

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Abstract

The 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway occurs in the plastids of higher plants and in most economically important prokaryotes where it is responsible for the biosynthesis of the isoprenoid building blocks, isopentenyl diphosphate and dimethylallyl diphosphate. These five-carbon compounds are the substrates for the enormous variety of terpenoid products, including many essential metabolites and substances of commercial value. Increased knowledge of the regulation of the MEP

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pathway is critical to understanding many aspects of plant and microbial metabolism as well as in developing biotechnological platforms for producing these commercially valuable isoprenoids. To achieve this goal, researchers must have the ability to investigate the in vivo kinetics of the pathway by accurately measuring the concentrations of MEP pathway metabolites. However, the low levels of these metabolites complicate their accurate determination without suitable internal standards. This chapter describes a sensitive method to accurately determine the concentrations of MEP pathway metabolites occurring at trace amounts in biological samples using liquid chromatography coupled to triple quadrupole mass spectrometry. In addition, simple protocols are given for producing stable isotope-labeled internal standards for these analyses.

1. INTRODUCTION

Isoprenoids, also known as terpenoids, are a very diverse group of compounds present in all living organisms with a multitude of different functions (Croteau, Kutchan, & Lewis, 2000; McGarvey & Croteau, 1995). Despite the structural diversity of this group, isoprenoids are all derived from the same five-carbon building blocks, isopentenyl diphosphate (IDP) and its isomer dimethylallyl diphosphate (DMADP). Isoprenoids have important functions in primary metabolism as electron carriers in respiration and photosynthesis (ubiquinone, plastoquinone), membrane components (sterols, cholesterol), photosynthetic pigments (carotenoids, chlorophylls), and plant growth regulators (abscisic acid, brassinosteroids, cytokinins, strigolactones). In secondary metabolism, an enormous variety of structures play a multitude of roles in defense against enemies, attraction of mutualists, and internal signaling (Bouvier, Rahier, & Camara, 2005; Croteau et al., 2000; Gershenzon & Dudareva, 2007; Rodriguez-Concepcion, 2006).

Isoprenoid biosynthesis is unusual in the fact that two completely different pathways each produce the basic five-carbon precursors IDP and DMADP (Eisenreich et al., 1998; Rohmer, Knani, Simonin, Sutter, & Sahm, 1993; Rohmer, Seemann, Horbach, BringerMeyer, & Sahm, 1996). The mevalonate (MVA) pathway was once thought to be only responsible for the biosynthesis of these precursors, but in the 1990s the methylerythritol 4-phosphate (MEP) pathway was discovered as an alternative route to IDP and DMADP (Cordoba, Salmi, & Leon, 2009; Eisenreich et al., 1998). Most organisms use only one of the two pathways for the biosynthesis of the isoprenoid precursors. The MVA pathway is exclusive to archaea, fungi, and animals, while the MEP pathway is present in most eubacteria and apicomplexan protozoa (like *Plasmodium falciparum*).

By contrast, plants, green algae, and some bacteria use both pathways (Lichtenthaler, Rohmer, & Schwender, 1997; Rodriguez-Concepcion & Boronat, 2002 Rohmer, 1999).

In contrast to the MVA pathway that involves sequential additions of acetyl-CoA units followed by reduction and phosphorylation, the MEP pathway starts with the synthesis of 1-deoxy-D-xylulose 5-phosphate (DXP) by condensation of glyceraldehyde 3-phosphate (G3P) and pyruvate (Pyr) via DXP synthase (DXS). DXP is then reduced by DXP reductoisomerase (DXR) to 2-C-methyl-D-erythitol 4-phosphate (MEP) that is converted into 2-C-methyl-D-erythritol-2,4-cyclodiphosphate (MEcDP) in three steps via the cytidyl conjugates, 4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol (ME-CDP) and ME-CDP phosphate (MEP-CDP) as intermediates. Finally, MEcDP is successively reduced to (*E*)-4-hydroxy-3-methylbut-2-enyl 4-diphosphate (HMBDP), catalyzed by HMBDP synthase (HDS), and then to a mixture of IDP and DMADP, catalyzed by HMBDP reductase (HDR), to complete the pathway (Hunter, 2007; Phillips, Leon, Boronat, & Rodriguez-Concepcion, 2008) (Fig. 1). Since the discovery of the MEP pathway, increasing attention has been paid to the individual intermediates both because of interest in pathway regulation and the finding that in plants some intermediates are also involved in complex signaling cascades from plastids (site of the MEP pathway in plants) to the nucleus (Gonzalez-Cabanelas et al., 2015; Ward, Baker, Llewellyn, Hawkins, & Beale, 2011; Xiao et al., 2012). This highlights the importance of developing analytical tools to detect and quantify MEP intermediates to investigate these and other possible functions.

The absence of the MEP pathway in animals, including humans, makes it an attractive target for the development of compounds, such as antibiotics and herbicides, targeted at organisms possessing the pathway (Testa & Brown, 2003). In addition, the commercial value of some isoprenoids as pigments (carotenoids), industrial materials (resins and rubbers), flavor and fragrances (monoterpenes), drugs (taxol, artemisinin), and even biofuels has triggered interest in enhancing their production (Rodriguez-Concepcion, 2006; Tippmann, Chen, Siewers, & Nielsen, 2013). During the last decade many attempts at engineering the MEP pathway have been made in order to increase the supply of IPP and DMAPP for the biosynthesis of valuable isoprenoids, but with few exceptions the results have been disappointing (Ajikumar et al., 2010; Klein-Marcuschamer, Ajikumar, & Stephanopoulos, 2007). In fact, when metabolic engineering of isoprenoid biosynthesis has proved successful in *Escherichia coli*, the native MEP pathway

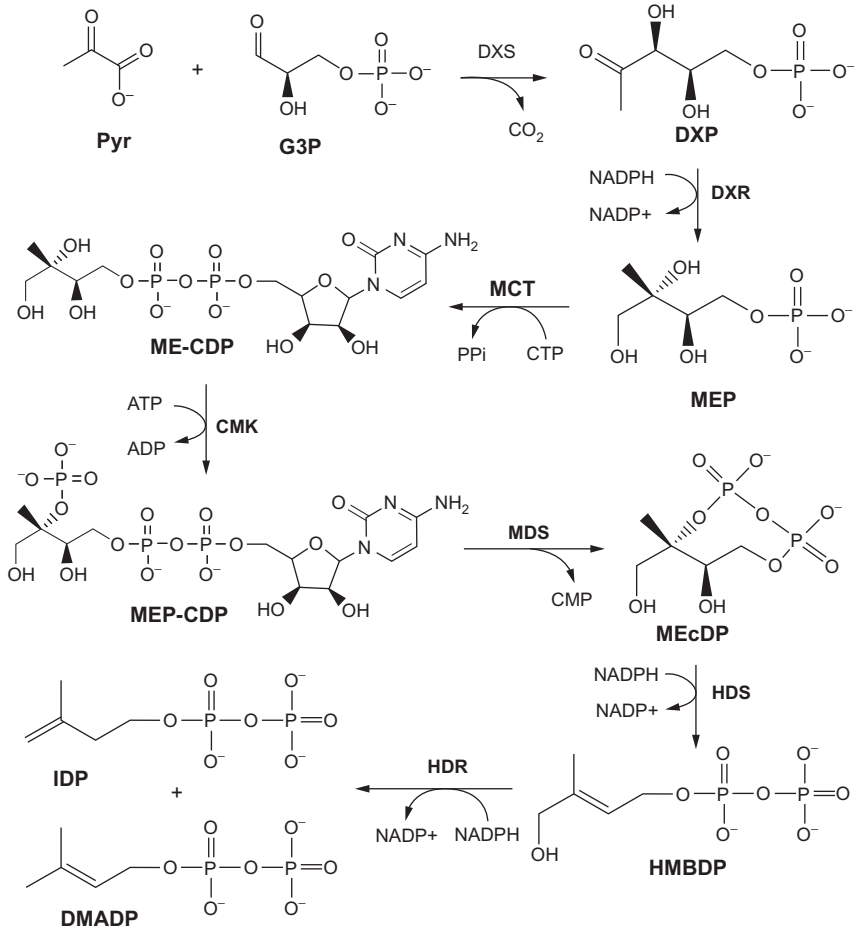

Fig. 1 The 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway. Pyruvate (Pyr) and glyceraldehyde 3-phosphate (G3P) are condensed into 1-deoxy-D-xylulose 5-phosphate (DXP) by DXP synthase (DXS) with a loss of carbon dioxide. DXP is reduced to MEP by DXP reductoisomerase (DXR) and the product reacts with cytidine triphosphate (CTP) to produce 4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol (ME-CDP) with a loss of a diphosphate (PPi) moiety. ME-CDP is converted to ME-CDP phosphate (MEP-CDP) by ME-CDP kinase (CMK) using adenosine triphosphate (ATP), which loses a phosphate to form adenosine diphosphate (ADP). MEP-CDP is cyclized via a loss of cytidine monophosphate (CMP) to 2-C-methyl-D-erythritol 2,4-cyclodiphosphate (MEcDP) by MEcDP synthase (MDS). MEcDP is reduced to (*E*)-4-hydroxy-3-methylbut-2-enyl diphosphate (HMBDP) by HMBDP synthase (HDS). HMBDP is reduced to a mixture of isopentenyl diphosphate (IDP) and dimethylallyl diphosphate (DMADP) by HMBDP reductase (HDR).

has been ignored and the MVA pathway ectopically expressed (Cervin, Whited, Chotani, Valle, & Fioresi, 2009; Tsuruta et al., 2009). However, it would actually be better to employ the MEP pathway for this purpose since it has a lower energy requirement than the MVA pathway when using only glucose and glycerol as carbon sources (Ajikumar et al., 2010; Steinbüchel, 2003). The lack of success in engineering the MEP pathway is often attributed to insufficient knowledge of its control (Wright et al., 2014).

The majority of the work done on the regulation of the MEP pathway has focused on the regulation of gene expression. However, some studies indicated that posttranscriptional and especially posttranslational regulation are also important in MEP pathway regulation (Flores-Perez, Sauret-Gueto, Gas, Jarvis, & Rodriguez-Concepcion, 2008; Guevara-Garcia et al., 2005; Kobayashi et al., 2007). In this context, measurements of enzyme activities and the accompanying pools of metabolites are needed to understand the operation of the pathway. Equally important is the in vivo measurement of flux. Since the MEP pathway leads to several major products, the only suitable approach to measure flux is to follow the incorporation rate of stable isotopic label into metabolites (Wright et al., 2014). Here we describe extraction, analysis, and quantification methods to measure intermediates of the MEP pathway, including their amounts of stable isotopic label. These procedures will further our understanding how the pathway works in different organisms, providing a foundation for increased production of isoprenoids with high commercial values.

2. PREPARATION OF STABLE ISOTOPE-LABELED INTERNAL STANDARDS

Stable isotope-labeled internal standards have identical physical and chemical properties to their target analytes but a different mass. Hence such standards show the same separation characteristics as the analyte during chromatography, but can be distinguished by mass spectrometry. The addition of stable isotope-labeled internal standards to a sample therefore facilitates the identification and absolute quantification of metabolites. Knowing the amount of standard added allows the measurement of the quantity of the analyte (recovery), the ion suppression effect of the matrix components, and mass detector fluctuations (Stokvis, Rosing, & Beijnen, 2005). However, stable isotope-labeled internal standards are very expensive and often, as is the case for the MEP pathway metabolites, not commercially available.

We therefore developed a method to produce such standards for MEP pathway intermediates by growing *E. coli* BL21(DE3) cells in M9 minimal medium using universally labeled ^{13}C-D-glucose as the carbon source.

2.1 Growing *E. coli* with Labeled Glucose as a Carbon Source

A survey was first conducted of *E. coli* cultures at various growth stages to determine at what stage MEP pathway metabolites are at the highest levels. A 5-mL starter culture of *E. coli* BL21(DE3) was grown in 50 mL M9 minimal medium and the MEP pathway metabolites were measured at regular intervals over 14 h at 37°C on a shaker set at 220 revolutions per minute (rpm). The highest concentrations of the metabolites were detected after 5–6 h of growth when the optical density at 600 nm (OD_{600}) was between 1.3 and 1.6 (Fig. 2). Harvesting cells at this stage grown with ^{13}C-labeled glucose as the carbon source, we obtained high levels of MEP pathway metabolites with an excess of 80% fully labeled ^{13}C-compounds and the remainder partially labeled; unlabeled metabolites were not detectable (Fig. 3).

1. *E. coli* BL21(DE3) was grown overnight in 5 mL LB medium at 37°C in a shaking incubator at 220 rpm. The next day the cells were centrifuged at 4200 × *g* for 5 min and the supernatant was removed. Removal of the LB medium is necessary to prevent the formation of unlabeled MEP pathway metabolites.
2. Resuspend cells in 50 mL M9 minimal medium containing [$^{13}C_6$]-D-glucose as carbon source and grow cells at 37°C in a shaking incubator at 220 rpm until an OD_{600} value between 1.3 and 1.6 is reached (5–6 h).
3. Centrifuge cells for 5 min at 4200 × *g* and 4°C, remove the supernatant, and add 5 mL ice-cold extraction solvent consisting of acetonitrile/methanol/water (2:2:1, v/v/v) containing 0.1% ammonium hydroxide to stop the metabolism and extract the MEP pathway intermediates. Vortex the cell suspension thoroughly and incubate on ice for at least 15 min with occasional vortexing.
4. Transfer the liquid phase to a new tube after centrifuging 5 min at 20,000 × *g* and 4°C and dry in a rotary evaporator. Alternatively the solvent can be separated into aliquots and evaporated under a stream of nitrogen gas at 40°C in a heating block or centrifugal vacuum evaporator at 45°C.
5. Dissolve residue in 500 μL 10 m*M* ammonium acetate, adjusted to pH 9 with ammonium hydroxide, centrifuge 5 min at 20,000 × *g* and 4°C, and transfer supernatant to a new 1.5-mL safe-lock microcentrifuge tube kept on ice.

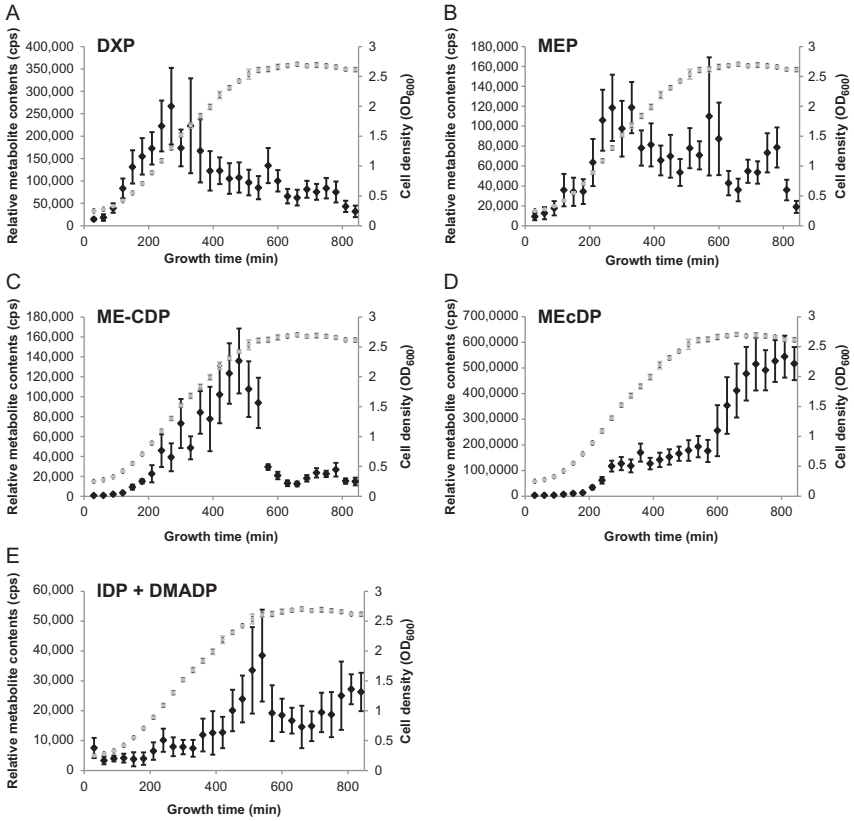

Fig. 2 The correlation between the content of MEP pathway metabolites and the growth phase of an *E. coli* culture. Shown are the average amount of metabolites from three different experiments (*solid symbols*), expressed as peak area of a 5 µL sample extract. The metabolites were extracted and analyzed as described in the text from 1 mL *E. coli* grown in M9 minimal medium incubated at 37°C and harvested at 30 min intervals. The bacterial cell density (*open symbols*) was measured by determining the optical density at 600 nm. Shown are the relative contents of (A) DXP, (B) MEP, (C) ME-CDP, (D) MEcDP, and (E) IDP + DMADP.

6. Extract with 500 µL chloroform by vortexing thoroughly. Centrifuge 5 min at 20,000 × *g* at 4°C, and remove the upper aqueous phase containing the stable isotope-labeled internal standards. Although the standards are stable for at least a month when stored at 4°C, we recommend making aliquots and drying under a steam of nitrogen gas for longer term storage. We typically dilute the preparation 1:9 (v/v) with extraction solvent or with the MEP pathway metabolite extract for absolute quantification.

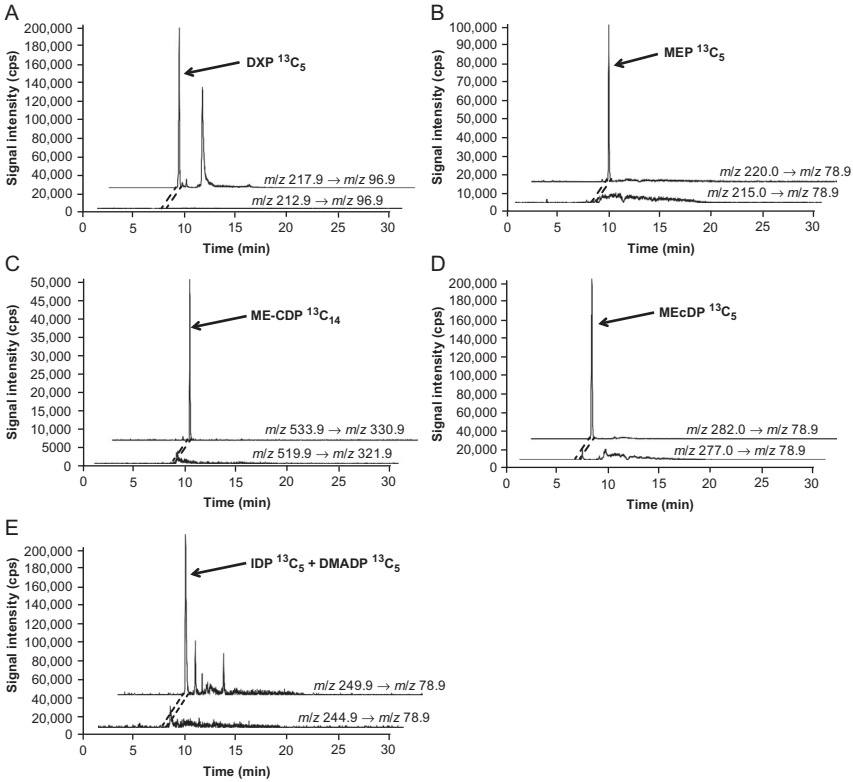

Fig. 3 Stable isotope-labeled MEP pathway intermediates extracted from *E. coli* grown at 37°C in M9 minimal medium with universally labeled glucose $^{13}C_6$ used as carbon source. The metabolites were analyzed with LC–MS/MS using electrospray ionization after separation on a HILIC column as described in the text. The selected reaction monitoring (SRM) of the fully labeled (A) DXP, (B) MEP, (C) ME-CDP, (D) MEcDP, and (E) IDP + DMADP are shown in the rear traces with the SRM of the unlabeled compound in the front traces.

2.2 Isolation of MEcDP by Export from Transgenic *E. coli*

Recent research has shown that the MEP pathway intermediate MEcDP also functions in stress responses in *Arabidopsis thaliana* (Gonzalez-Cabanelas et al., 2015; Xiao et al., 2012). However, research on the additional roles of MEcDP is hampered by the excessive cost of this metabolite from commercial suppliers. Thus we have developed a rapid and simple method to produce MEcDP at low cost that exploits the tendency of transgenic *E. coli* to export MEcDP to the medium with increased flux through the MEP pathway (Zhou, Zou, Stephanopoulos, & Too, 2012). The first

enzyme in the MEP pathway, DXS, is the major flux controlling enzyme (Wright et al., 2014). By overexpressing DXS in *E. coli* BL21(DE3) cells, we increased pathway flux and export of MEcDP to the medium. This results not only in greater amounts of MEcDP but its occurrence in the medium simplifies its purification. After concentrating the MEcDP through freeze-drying, this metabolite was purified using chromatography on microcrystalline cellulose.

1. Start a small 5 mL culture of transformed *E. coli* BL21(DE3) cells overexpressing DXS in LB medium and grow it overnight at 37°C shaking at 220 rpm.
2. Add the 5 mL starter culture to 45 mL LB medium and grow at 37°C and 220 rpm shaking until an OD_{600} between 0.4 and 0.5 is reached when the cells are in exponential growth. Induce the expression of DXS cloned in the pH 9 plasmid (Yu & Liu, 2006) by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 m*M* and let the cells grow under the same conditions for another 3 h. IPTG induction is necessary due to the T7 promoter used on the pH 9 plasmid.
3. Replace the LB medium with M9 minimal medium by centrifuging the cells for 5 min at $4200 \times g$ at 28°C, removing the old medium and resuspending the cells in M9 medium with universally labeled ^{13}C-D-glucose as carbon source. Incubate the cells at 28°C and 220 rpm.
4. Lyophilize the supernatant after removing the cells by centrifuging for 5 min at 4°C at $4200 \times g$. Resuspend the pellet in 5 mL of isopropanol/methanol/water (2:2:1, v/v/v) containing 0.1% formic acid. Centrifuge at $4200 \times g$ for 5 min at 4°C to remove any precipitate.
5. Load the sample on a microcrystalline cellulose column with a diameter of 10 mm and a length of 600 mm and elute with isopropanol/methanol/water (2:2:1, v/v/v) containing 0.1% formic acid at a flow rate of 1.0 mL min^{-1}. Fractions of 4 mL can then be collected and analyzed for MEcDP content using direct injection into the triple quadrupole mass spectrometer (MS/MS). Inject a 1-μL portion of the collected fraction into a solvent flow of 1 mL min^{-1} connected to the ion source of the MS/MS. The solvent used is the same as was used for the running solvent on the cellulose column. The parameters used for detection with the mass spectrometer are described in Section 4.2. Combine the fractions containing MEcDP and evaporate the solvent using a rotary evaporator to obtain the purified MEcDP. Using this approach we could purify approximately 2 mg MEcDP with an NMR determined purity of 80%. If more MEcDP is needed, the culture volume can be scaled up.

3. EXTRACTION OF METHYLERYTHRITOL PHOSPHATE PATHWAY INTERMEDIATES FROM BIOLOGICAL SOURCES

As a primary pathway with a direct connection to photosynthesis (Li & Sharkey, 2013), the MEP pathway has a rapid flux rate and so care must be taken to quench metabolic activity as soon as possible after harvest in order to obtain accurate measurements of metabolic pools. For example, wild-type *A. thaliana* has a MEP pathway flux of 3.44 pmol mg^{-1} min^{-1} when grown at 21°C at a light intensity of 140 μmol m^{-2} s^{-1} photosynthetic photon flux density. Hence to have an effect of less than 5% on a typical amount of ~20 pmol mg^{-1} DXP (Wright et al., 2014), metabolism should be halted within 20 s. This is usually accomplished by freezing in liquid nitrogen.

Another consideration in the extraction of MEP pathway metabolites is choosing a buffer to maximize their stability. Since nearly all of these metabolites are phosphorylated, a slightly alkaline pH of 9 was selected to maintain ionization for increased stability and solubility. At higher pH values, there is a risk of degrading MEcDP by cleaving the cyclodiphosphate linkage (Ge, d'Avignon, Ackerman, & Sammons, 2012).

3.1 Extraction of Methylerythritol Phosphate Pathway Intermediates from Plant Materials

1. For fast quenching of metabolism material was directly immersed in liquid nitrogen after harvest. For typical pathway flux (Wright et al., 2014), quenching within 20 s should be adequate for accurate, reproducible analyses. However with more rapid flux or smaller pool sizes, the time window necessary to deactivate metabolism might need to be reduced further. In such cases, it may be advisable to flash-freeze the plant material while still under the light source (Arrivault et al., 2009). Once frozen, the next step is to homogenize the plant material to obtain a fine powder. This can be done using a mortar and pestle precooled in liquid nitrogen. An additional method is to first freeze-dry the material and then homogenize it in a vertical oscillating ball mill. Most important is that the homogenized material be a fine powder without any distinguishable particles to assure full extraction of the metabolites.
2. For extraction, weigh approximately 5 mg of dried plant material into a 1.5-mL safe-lock microcentrifuge tube, taking care to note the exact weight. This weight will be used later to normalize the quantified

compound amounts to the weight of plant material extracted. A weight within 0.5 mg of the intended 5 mg gives maximum extraction efficiency with the amounts of extraction solvents listed. Add 250 μL of an acetonitrile–10 m*M* ammonium acetate pH 9 mixture (1:1, v/v) and vortex thoroughly. The acetonitrile in the extraction solvent helps to dissolve membranes and is needed for maximum extraction of the metabolites from chloroplasts.

3. After centrifugation for 5 min at 20,000 × *g* in a microcentrifuge, 200 μL of the supernatant is transferred to a new 1.5-mL safe-lock microcentrifuge tube. For maximum efficiency, the pellet should be extracted with another 250 μL of extraction solvent as described in step 2.
4. The supernatants are then combined and the extract evaporated under a stream of nitrogen in a 40°C heating block. Alternatively the supernatant can be dried in a centrifugal vacuum evaporator at 45°C.
5. Dissolve the residue in 100 μL of 10 m*M* ammonium acetate, adjusted to pH 9 with ammonium hydroxide, by thorough vortexing and add 100 μL of chloroform to extract the hydrophobic compounds from the sample. This is to prevent unnecessary contamination of the hydrophilic interaction liquid chromatography (HILIC) column and mass spectrometer. The extract is then centrifuged for 5 min at 20,000 × *g* to facilitate fast phase separation.
6. Transfer 50 μL of the aqueous phase to a new 1.5-mL safe-lock microcentrifuge tube; add 50 μL acetonitrile, vortex, and centrifuge 5 min at 20,000 × *g* to remove any precipitate. The supernatant is then analyzed by liquid chromatography-triple quadrupole mass spectrometry (LC–MS/MS) as described in Section 4 or stored at –20°C for later analysis. In the presence of isotopically labeled internal standards, the extract can be stored for an extended period without detectable losses.

3.2 Extraction of Methylerythritol Phosphate Pathway Intermediates from Bacterial Cultures

The methylerythritol phosphate pathway is present in most eubacteria, including *E. coli*, as an essential biosynthetic route. Due to the absence of a rigid cell wall, we extract the bacteria cells with a mixture of ice-cold acetonitrile, methanol, and water to break the cell membrane and quench metabolic processes. The *E. coli* cultures were grown in 50 mL M9 minimal medium. Extraction proceeds as follows:

1. Collect *E. coli* cells from a 1 mL growth culture and centrifuge at 20,000 × *g* for 5 min at 4°C to precipitate the cell pellet.

2. Freeze the sample in liquid nitrogen and store at –20°C for later extraction, or extract the cells directly by adding 1 mL of ice-cold acetonitrile/methanol/water (2:2:1, v/v/v) containing 0.1% ammonium hydroxide. Incubate on ice for at least 15 min with occasional vortexing to facilitate membrane breakage and maximum extraction efficiency.
3. Transfer the liquid phase to a new 1.5-mL safe-lock microcentrifuge tube after centrifuging for 5 min at 20,000 × *g*. Dry under a stream of nitrogen gas at 40°C in a heating block or centrifugal vacuum evaporator at 45°C.
4. Dissolve the residue in 100 μL 10 m*M* ammonium acetate, adjusted to pH 9 with ammonium hydroxide, by thorough vortexing and centrifuge 5 min at 20,000 × *g*. Transfer 90 μL of the supernatant to a new 1.5-mL safe-lock microcentrifuge tube.
5. Extract with an equal volume of chloroform and separate the phases by centrifugation at 20,000 × *g*. Transfer 50 μL of the upper aqueous phase to a new 1.5-mL safe-lock microcentrifuge tube; add 50 μL acetonitrile, vortex, and centrifuge at 20,000 × *g* for 5 min to remove any suspended particles. Transfer the supernatant to an HPLC vial for LC–MS/MS analysis as described in Section 4 or store at –20°C for later analysis. In the presence of isotopically labeled internal standards, the extract can be stored for an extended period without detectable losses.

4. ANALYSIS OF METHYLERYTHRITOL PHOSPHATE PATHWAY METABOLITES BY LC–MS/MS

Mass spectrometry furnishes a very sensitive, information-rich platform for analyzing most metabolites, but to take full advantage it is necessary to optimize the chromatographic separation. This is particularly true for phosphorylated analytes, such as the MEP pathway metabolites, not only to improve sensitivity but also to minimize overlap with other phosphorylated compounds with similar masses that are also likely to experience similar fragmentations due to the facile loss of the phosphate groups. Separation of highly hydrophilic phosphorylated compounds is typically performed using counter ion liquid chromatography (Luo, Groenke, Takors, Wandrey, & Oldiges, 2007) or ion exchange liquid chromatography (Lunn et al., 2006). However, in counter ion chromatography mass spectrometry signals are reduced due to ion suppression and contamination of the mass spectrometer by the counter ion (Rutters, Mohring, Rullkotter, Griep-Raming, & Metyger, 2000). Ion exchange chromatography requires a membrane-based

electrolytic suppressor when coupled to a mass spectrometer to continuously neutralize pH and remove salts prior to infusion (Rabin, Stillian, Barreto, Friedman, & Toofan, 1993), which is not practical for multipurpose instruments and entails additional costs.

HILIC is an attractive alternative to separate small polar compounds (Li & Sharkey, 2013). HILIC employs polar stationary phases (Olsen, 2001; Oyler et al., 1996) and mobile phase solvents containing a water miscible organic solvent with a small amount of water. The low water content in the mobile phase results in a liquid–liquid extraction system with an aqueous layer around the surface of the polar stationary phase and an organic layer elsewhere. Increasing the aqueous content of the mobile phase causes the elution of compounds that had been retained in the water layer associated with the stationary phase (Buszewski & Noga, 2012; Mateos-Vivas, Rodriguez-Gonzalo, Garcia-Gomez, & Carabias-Martinez, 2015) through a partitioning effect. Silica is the most widely used chromatographic support in HILIC and can be modified by the binding of a variety of functional groups, such as aminopropyl, amide, or cyano moieties (Ikegami, Tomomatsu, Takubo, Horie, & Tanaka, 2008). A typical mobile phase employed in HILIC retention contains between 70% and 90% of acetonitrile (Alpert, 1990; Naidong, 2003), which provides enough water for the creation of the aqueous layer and the complete solubilization of the hydrophilic compounds from the sample.

4.1 Separation by HILIC

To choose the best HILIC stationary phase to separate and detect the MEP pathway metabolites, we tested columns of Nucleodex β-OH (5 μm, 200 × 4.0 mm; Macherey-Nagel) (Pulido, Toledo-Ortiz, Phillips, Wright, & Rodriguez-Concepcion, 2013), Kinetex HILIC (2.6 μm, 150 × 2.1 mm; Phenomenex), Zorbax RX-SIL (5 μm, 150 × 2.1 mm; Agilent), Atlantis HILIC (3 μm, 150 × 2.1 mm; Waters) (Ghirardo et al., 2014), ZIC-pHILIC (5 μm, 150 × 2.1 mm, Merck) (Wright et al., 2014), XBridge BEH Amide (3.5 μm, 150 × 2.1 mm; Waters) (Gonzalez-Cabanelas et al., 2015; Saladie, Wright, Garcia-Mas, Rodriguez-Concepcion, & Phillips, 2014; Wright & Phillips, 2014), and XBridge BEH Amide XP (2.5 μm, 150 × 2.1 mm; Waters). The best results were obtained with the XBridge BEH Amide XP column which uses an ethylene-bridged hybrid silica gel support prepared from tetraethoxysilane and bis(triethoxysilyl)ethane with trifunctional-bonded amide as functional group.

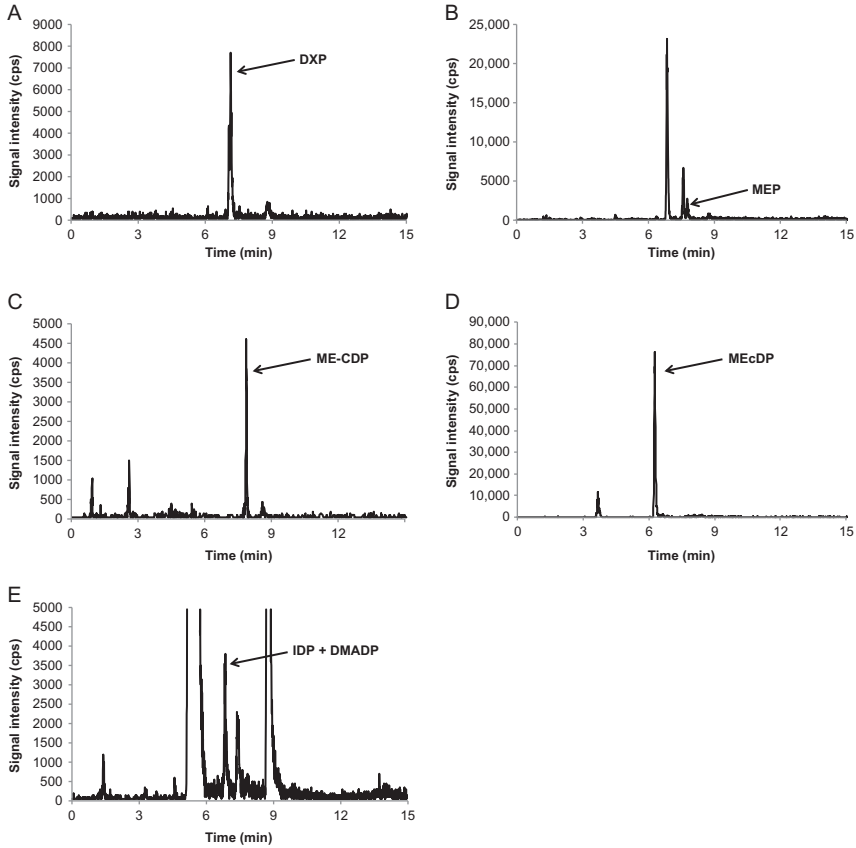

Fig. 4 MEP pathway intermediates extracted from a mature *A. thaliana* rosette. The metabolites were analyzed with LC–MS/MS using electrospray ionization after separation on a HILIC column as described in the text. The selected reaction monitoring (SRM) of (A) DXP, (B) MEP, (C) ME-CDP, (D) MEcDP, and (E) IDP + DMADP with the peaks representing the MEP pathway metabolites indicated by an *arrow*.

The version with 2.5 μm resin particle size was chosen to better separate the metabolic intermediate MEP from various coeluting compounds (Fig. 4). The column was run at a high flow rate of 500 μL min^{-1} relative to the small 2.1-mm column diameter (van Deemter, Zuiderweg, & Klinkenberg, 1956) to improve separation efficiency. This leads to column back pressures of up to 500 bar, which are too high for standard HPLC systems. In the absence of a high-pressure HPLC instrument, we recommend using the XBridge BEH Amide column with a 3.5-μm particle size which runs at a maximum back pressure of around 220 bar under similar flow rates.

The mobile phase was selected to reduce peak broadening and tailing. The diphosphorylated compounds, IDP and DMADP, showed especially pronounced peak tailing caused by hydrogen bonding between the phosphate groups and free silanol groups of the silica in the resin (Gritti, Kazakevich, & Guiochon, 2007) as well as interaction with metals in the LC–MS/MS system (Tuytten et al., 2006). Hydrogen bonding was minimized by using an end-capped stationary phase of the XBridge BEH Amide XP column. Interaction with the metal contained in the instrumental setup was reduced by using ammonium bicarbonate as a buffer additive in the mobile phase (Asakawa et al., 2008; Guo & Gaiki, 2005), and by increasing the pH (Preinerstorfer, Schiesel, Lammerhofer, & Lindner, 2010). Increasing the pH by more than 1 unit above the pK_a of the analytes has the additional benefit of stabilizing their charge (Boersema, Mohammed, & Heck, 2008; Buszewski & Noga, 2012). However, the high pH led to decreased retention due to repulsion between the negatively charged phosphates and the residual negative charge of the stationary phase (Alpert, 1990). To increase the retention, we increased the ammonium bicarbonate to 20 m*M* (the maximum advisable concentration for liquid chromatography with mass spectrometry detection) to decrease the repulsion between analytes and the stationary phase (Asakawa et al., 2008) and to increase the volume or hydrophilicity of the aqueous layer (Mateos-Vivas et al., 2015). To increase analyte retention further, a polar cationic base was added to the mobile phase to form hydrophilic ion pairs with the phosphate groups and so further reduce electrostatic repulsion and increase retention (Mateos-Vivas et al., 2015). This was ammonium hydroxide added until a pH of 10.5 was reached.

Another advantage of HILIC separation in comparison with other chromatographic methods is the generally small effect of temperature on retention properties (Buszewski & Noga, 2012). Our analysis did not show marked differences in the retention times or in the quality of the chromatograms under different temperatures so to promote column lifetime we used a temperature of 25°C.

The optimized separation conditions used the XBridge BEH Amide XP Column (2.5 μm, 150 × 2.1 mm; Waters) together with a guard column containing the same sorbent (2.5 μm, 10 × 2.1 mm) and a SSI^{TM} high-pressure precolumn filter (Sigma-Aldrich). The mobile phases were 20 m*M* ammonium bicarbonate adjusted to pH 10.5 with LC–MS grade ammonium hydroxide as solvent A, and 80% acetonitrile containing 20 m*M* ammonium bicarbonate adjusted to pH 10.5 with LC–MS grade ammonium hydroxide

Table 1 Liquid Chromatography Solvent Gradient Profile for Separation of Methylerythritol Phosphate Pathway Intermediates on the XBridge BEH Amide XP HILIC Column

Time (min)	% Solvent A[a]	% Solvent B[b]
0	0	100
5	16	84
10	16	84
11	40	60
15	40	60
15.10	0	100
30	0	100

[a]Solvent A is 20 m*M* ammonium bicarbonate, pH 10.5, adjusted with LC–MS grade ammonium hydroxide
[b]Solvent B is 80% acetonitrile with 20 m*M* ammonium bicarbonate adjusted to pH 10.5 with LC–MS grade ammonium hydroxide.
The column temperature was 25°C with a flow rate of 500 μL min^{-1} and 5 μL sample injected.

for solvent B. The solvent gradient profile is described in Table 1, with steps of elution (0–10 min), column wash (10–15 min), and column equilibration (15–30 min). Separation was performed at 25°C with a flow rate of 500 μL min^{-1}, and the volume injected was 5 μL. Fig. 4 shows a typical chromatogram of MEP metabolites extracted from *A. thaliana* in which six of the eight metabolites of the MEP pathway are detected and quantified. Of the metabolites that cannot be quantified, MEP-CDP is very labile and spontaneously breaks down to ME-CDP, while HMBDP occurs at levels below our limit of quantification (LOQ).

4.2 Detection by Tandem Mass Spectrometry

The mobile phase used for HILIC is especially suitable for standard electrospray ionization mass spectrometry because of its high volatility (Ikegami et al., 2008). The use of mass spectrometry detection has long been appreciated for metabolite analyses because of its high sensitivity and ability to separate coeluting compounds based on mass measurements. For pathway studies, mass spectrometric detection also facilitates measurements of stable isotope-labeled compounds which can be used for absolute quantification of intermediate pools and for label incorporation experiments for determination of flux. Tandem mass spectrometry in particular is extremely useful for detection against the complexity of plant or

Table 2 Ion Source Parameter Settings Used for Ionizing the MEP Pathway Metabolites in the Ion Source of an API 5000 Tandem Mass Spectrometer (AB Sciex)

Ion Source Parameter	Optimized Setting
Ion spray voltage	–4500 V
Ion source temperature	700°C
Nebulizer gas	70 psi
Heating gas	30 psi
Curtain gas	30 psi

bacterial matrices. Using the widely available API 5000 triple quadrupole mass spectrometer (AB Sciex), the ion source parameters were optimized for all of the MEP pathway intermediates under investigation simultaneously (Table 2). The ion source was used in the negative ionization mode with nitrogen gas for the nebulizing, heating, and curtain gas. The results should be applicable to all mass spectrometers from this manufacturer, since all these use the same ion source.

The individual intermediates were quantified on a triple quadrupole mass analyzer by multiple reaction monitoring using specific precursor ion—product ion reactions for each metabolite. The optimized parameters for each reaction are given in Table 3. We also give parameters for quantifying stable isotope-labeled standards of each metabolite. With the molecular ion as a precursor, most of the MEP pathway metabolites gave either m/z 96.9 or m/z 78.9 as the most sensitively detected product ion after fragmentation, and these were used for quantification. These masses both correspond to a fragment from the phosphate group of the precursor ion, with m/z 96.9 representing $[\text{phosphate–H}]^-$ and m/z 78.9 representing $[\text{phosphate–H}_2\text{O–H}]^-$. These phosphate fragment ions are the only detectable product ions for MEP, MEcDP, and IDP/DMADP and the most sensitively detected for DXP. Use of the phosphate fragment as product ions for quantification is also advantageous when measuring the incorporation of ^{13}C because of the reduced scan combinations necessary. Since the product ion does not contain any carbons, only the mass increase in the precursor ion needs to be measured for determining the amount of ^{13}C in the metabolites. For the MEP pathway intermediate ME-CDP, pronounced background noise was observed when monitoring the phosphate product ion, and therefore we used the m/z 321.9 ion as product ion for quantification which represents the cytidine diphosphate fragment.

Table 3 Parameters for Quantifying Individual MEP Pathway Metabolites and Their Respective Stable Isotopically Labeled Internal Standards by Tandem Mass Spectrometry with a Triple Quadrupole Analyzer

Compound	Retention Time (min)	Precursor Ion → Product Ion	Declustering Potential (V)	Collision Energy (V)	Cell Exit Potential (V)
DXP	7.1	212.9 → 96.9	–60	–16	–15
DXP-$^{13}C_5$	7.1	217.9 → 96.9	–60	–16	–15
MEP	7.8	215.0 → 78.9	–70	–36	–11
MEP-$^{13}C_5$	7.8	220.0 → 78.9	–70	–36	–11
ME-CDP	7.9	519.9 → 321.9	–105	–30	–9
ME-CDP-$^{13}C_{14}$	7.9	533.9 → 330.9	–105	–30	–9
MEcDP	6.3	277.0 → 78.9	–50	–38	–11
MEcDP-$^{13}C_5$	6.3	282.0 → 78.9	–50	–38	–11
IDP and DMADP	6.8	244.9 → 78.9	–45	–24	–11
IDP-$^{13}C_5$ and DMADP-$^{13}C_5$	6.8	249.9 → 78.9	–45	–24	–11

The retention times for the different metabolites as well as the masses of the precursor and product ions are also shown. Nitrogen was used as collision gas at a CAD setting of 10 and the entrance potential was set at –10 V for all metabolites.

4.3 Quantification

The accurate determination of analyte abundance by liquid chromatography-tandem mass spectrometry is affected by a range of factors, such as the stability of the molecule, recovery from the sample, degree of ion suppression, and limits of detection and quantification. The sample matrix is frequently observed to reduce the ionization efficiency of specific analytes due to other molecules that coelute with the compound of interest (Tang & Kebarle, 1993). In the analysis of MEP pathway intermediates, the use of a HILIC column should diminish ion suppression because of the high content of organic solvents which evaporate faster and so promote ionization of solvated analytes. The use of the negative ionization mode might also decrease ion suppression since fewer compounds are ionized under this mode than under positive ionization. Other strategies to reduce ion suppression include sample dilution or a decrease in injection volume to reduce the amount of interfering compounds.

To compensate for ion suppression, authentic standards are necessary. Stable isotope-labeled standards are ideal for this purpose because they behave similarly to the unlabeled analytes during the sample preparation, chromatography, and the ionization process (Van Eeckhaut, Lanckmans, Sarre, Smolders, & Michotte, 2009), and can be distinguished from the sample compounds based on their mass. Yet their expense or availability is often a major barrier to use. We have described methods for producing affordable stable isotope-labeled standards of MEP metabolites in Section 2.1 (Fig. 3). Their addition allows us to compensate not only for ion suppression but also for the loss of analytes during extraction and variations in instrument performance (Table 4) (Birkemeyer, Luedemann, Wagner, Erban, & Kopka, 2005). Optimization of the concentrations of stable isotopically labeled internal standards themselves is also necessary to avoid possible ion suppression effects. The concentrations of such standards should be kept in the same range as the natural occurrence of the sample compounds and in the linear range of the calibration curve (Bakhtiar & Majumdar, 2007; Freitas, Gotz, Ruff, Singer, & Muller, 2004). To be able to fully exploit an analytical method, it is necessary to know the limits of detection (LOD) and LOQ (Table 4). LOD is the lowest analyte concentration that can be detected, but not necessarily quantified, while the LOQ is the lowest concentration of an analyte that can be determined with acceptable precision and accuracy (Armbruster & Pry, 2008).

Table 4 Extraction Efficiency, Ion Suppression, Limit of Detection (LOD) and Limit of Quantification (LOQ) for the Analysis of the MEP Pathway Intermediates DXP, MEP, ME-CDP, MEcDP, and IDP/DMADP in *Arabidopsis thaliana* Leaf Extracts as Described in This Chapter

	DXP	MEP	ME-CDP	MEcDP	IDP/DMADP
Extraction efficiency (%)	74.5 ±3.0	62.9 ±1.9	78.0 ±2.5	83.9 ±3.2	24.2 ±1.9
Ion suppression (%)	65.7 ±2.7	64.5 ±2.9	75.1 ±4.0	55.3 ±3.5	58.3 ±2.3
LOD (pmol mL^{-1})	4.73 ±0.52	19.83 ±1.38	1.61 ±0.01	27.17 ±0.42	0.78 ±0.02
LOQ (pmol mL^{-1})	7.88 ±0.86	33.05 ±2.3	2.68 ±0.01	45.28 ±0.71	1.29 ±0.03

The extraction efficiency and ion suppression values represents the average ± SE ($n = 10$). The LOD was taken as the amount of metabolite detected that gave a signal-to-noise (S/N) ratio of 3 and the LOQ was taken as the amount necessary to give an S/N ratio of 5.

5. DISCUSSION AND SUMMARY

Other methods have been described for analyzing the MEP pathway metabolites from biological samples, most of which also use a sensitive mass spectrometer for detection. A notable exception is the measurement of MEcDP in plants under stress conditions with ^{31}P-NMR spectroscopy. Oxidative stress caused by high temperature and light intensities inhibits HDS that results in the drastic accumulation of the HDS substrate MEcDP to levels detectable by NMR (Rivasseau et al., 2009). When MEP pathway metabolites have been analyzed from crude extracts of biological samples, liquid chromatography separations are usually done by either counter ion liquid chromatography (Balcke, Bennewitz, Zabel, & Tissier, 2014; Zhang et al., 2011; Zhou et al., 2012) or HILIC (Baidoo, Xiao, Dehesh, & Keasling, 2014; Li & Sharkey, 2013). The mass analyzers include TOF (Baidoo et al., 2014; Perez-Gil et al., 2012; Zhou et al., 2012), quadrupole MS (Baidoo et al., 2014), MS/MS (Li & Sharkey, 2013; Zhang et al., 2011), and tripleTOF mass spectrometers (MS^2-QTOF) (Balcke et al., 2014). MS^2-QTOF instruments are a hybrid between MS/MS and TOF, where the third quadrupole is replaced with a TOF. This configuration allows for the accurate mass detection of the precursor ion as well as untargeted mass spectra determination of compounds through the sequential window acquisition of all theoretical mass spectra (SWATH) technique (Balcke et al., 2014).

However, none of these previous methods directly measure the recovery efficiency and ion suppression during analysis. Technical variation during extraction procedures and ion suppression fluctuations due to slight differences in the matrix of different samples can lead to decreased accuracy and precision. The addition of stable isotopically labeled internal standards is an excellent remedy for these problems, more effective than the use of external standard curves. Although protocols have been published for producing isotopically labeled standards of MEP metabolites (Hecht et al., 2001; Illarionova et al., 2006; Schuhr et al., 2001), they require tackling the intermediates one at a time using some sophisticated synthetic protocols. The protocol furnished here provides a method for producing stable isotope-labeled internal standards of the MEP pathway using only equipment and resources normally present in biochemical laboratories. These standards have facilitated the quantification of six of the eight MEP pathway intermediates in the model plant *A. thaliana*, a species that accumulates only low quantities of specialized isoprenoids. Thus the methods should be applicable to a range of plant and microbial taxa regardless of their level of isoprenoid production.

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