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A single-vial analytical and quantitative gas chromatography–mass spectrometry assay for terpene synthases

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Abstract

A quantitative assay for the analysis of sesquiterpene synthases, wherein each reaction mixture is formulated in glass gas chromatography vials, overlaid with organic solvent such as ethyl acetate, and subsequently vortexed to extract hydrocarbon reaction products into the organic phase after a suitable incubation period, was developed. The product-enriched organic phase is then sampled in an automated fashion and injected directly into a gas chromatograph–mass spectrometer without further workup for analysis and quantification of hydrocarbon products. Application of the vial assay to the analysis of amorpha-4,11-diene synthase (ADS), a sesquiterpene synthase, demonstrated the sensitivity of the assay for detection of major and minor reaction products and most notably for the identification of several sesquiterpene products that had escaped previous detection. A steady-state kinetic analysis of tobacco 5-epi-aristolochene synthase (TEAS), another sesquiterpene synthase, validated the quantitative nature of the assay, providing an alternative means to the established method of using radiolabeled substrate, extraction, and scintillation counting. This simplified assay provides a standardized method to facilitate analysis of terpene synthases and diverse mutant enzyme libraries by supplanting the common practice of using larger scale reactions, multiple extractions, and evaporative concentration of the organic phase prior to gas chromatography–mass spectrometry (GC-MS) analysis.

Terpenoids constitute the most prevalent and diverse family of natural products known, mediating myriad processes ranging from pathogen defense to pollinator attraction. Such biodiversity translates to applications of terpene natural products such as anticancer drugs, antibiotics, pesticides, flavorings, and fragrances [1]. Terpene synthases (cyclases) are the biosynthetic enzymes responsible for catalyzing the ionization of isoprenoid pyrophosphates (diphosphates), guiding the resulting acyclic and achiral carbocations through complex transformations into multicyclic and multichiral products [2]. Sesquiterpene synthases (cyclases) convert the 15-carbon isoprenoid substrate farnesyl-pyrophosphate (FPP)[1] into more than 300 distinct carbon skeletons. Downstream enzymes, particularly cytochrome P450s, often modify these hydrocarbon scaffolds, leading to thousands of oxygenated derivatives [3].

Among the variety of approaches used to study terpene cyclases, gas chromatography–mass spectrometry (GC–MS) has proven to be particularly useful given the volatility of the terpene hydrocarbon products. The principal application of this technique has been product

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[1] *Abbreviations used:* FPP, farnesyl-pyrophosphate; GC–MS, gas chromatography–mass spectrometry; ADS, amorpha-4,11-diene synthase; TEAS, tobacco 5-epi-aristolochene synthase; DTT, dithiothreitol; TIM, total ion monitoring; EDTA, ethylenediamine tetraacetic acid; KOH, potassium hydroxide; GC, gas chromatograph; MSD, mass selective detector; TIC, total ion current; SIM, selected ion monitoring; RSD, relative standard deviation.

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detection and identification, which is a fundamental aspect of the characterization of newly cloned or mutagenized cyclases [4–6]. Therefore, quantitative GC–MS analysis is critical in ascribing function to gene products and in defining functional features of these enzymes. Furthermore, most sesquiterpene cyclases generate one dominant product (~80% of the total terpenes produced) along with traces of a varying numbers of minor products. Interestingly, these less abundant reaction products often provide clues as to the cyclization route of the ionized FPP as it transforms into the dominant reaction product [7–9].

Previously, methods for the analysis of terpene cyclases typically were conducted on a milliliter scale using micro- to milligram levels of protein with substrate concentrations ranging from 20 to 50 μM [10]. Isolation of the resultant hydrocarbon products most often involved multiple two-phase extractions with pentane or hexane. The pooled organic phases were then passed over silica to trap and fractionate hydroxylated products, while the nonhydroxylated species could be concentrated slowly by rota-evaporation in an ice bath prior to the injection of the concentrated extract into the GC–MS.

Although this "standard" technique has been used successfully to characterize the reaction products of several terpene synthases, the labor required for more extensive experiments involving large collections of synthases or reaction conditions imposes practical limits on the sample size that can be analyzed and on the sensitivity and quantification attainable from these collections. Information gained from numerous functional studies [11] and hypotheses generated from the earliest crystal structures of terpene cyclases [12,13] provoke numerous questions centered on the structural basis underlying the reaction mechanism and rules governing substrate and product specificity in cyclases. The ability to address many of these questions in a global fashion is made possible by recent advances in the combinatorial synthesis of mutant and chimeric gene libraries [14,15], but the analysis of such libraries necessitates the development of more streamlined and sensitive assay methodologies.

The objective of the current study was to develop and qualify a standardized assay method to increase the throughput and allow quantitative analysis of terpene synthases by GC–MS. An assay that uses microliter-scale reaction volumes containing low microgram levels of purified proteins and micromolar substrate concentrations was developed. After reactants are combined in single vials, the reaction mixtures are overlaid with organic solvent, incubated, and then vortexed to extract hydrocarbons prior to GC–MS analysis. Unlike previously reported assays, the direct sampling of the organic phase without additional intervention ensures greater recovery of all reaction products and quantitative detection of products using GC–MS analysis. Although specifics surrounding automated sampling and analysis will depend on the available instrumentation, the sampling depth of the injection syringe can be adjusted to retrieve product most commonly from the organic phase. This process and the subsequent analysis can be conducted unattended, and the resultant data (gas chromatography trace and mass spectra) are readily stored for later analysis and/or incorporation into a database.

The effectiveness and sensitivity of this semi-automated procedure was demonstrated by its application to amorpha-4,11-diene synthase (ADS) product analysis. Previous analysis of ADS by standard procedures identified nine reaction products, three of which bore hydroxyl groups [16]. GC–MS analysis of products obtained using the single-vial reaction/extraction method identified all nine products previously observed plus an additional five products that were previously undetected. The potential of this technique for quantitative applications was established by the steady-state kinetic analysis of tobacco 5-epi-aristolochene synthase (TEAS). The kinetic parameters derived from this GC–MS analysis closely matched previous values obtained using a radiometric assay. Together, the work described here advances the ability to test hypotheses concerning terpene synthase mechanisms and evolution and greatly accelerates the pace at which structurally guided mutant libraries can be characterized for the identification of novel biosynthetic enzymes for academic and commercial applications.

Materials and methods

Materials

Farnesyl diphosphate (or FPP) was purchased from Echelon Biosciences as a powder and resuspended in 25 mM NH_4CO_3 as a 50-mM stock solution. The sesquiterpene standards (–)-α-cedrene, valencene, and *trans*,*trans*-farnesol, as well as organic solvents, were purchased from Sigma–Aldrich. Screw-cap glass vials, screw caps with Teflon-coated septa, and all GC–MS equipment were purchased from Agilent Technologies. All handling of organic solvents and sesquiterpenes was conducted in a fume hood.

Protein expression and purification

TEAS and ADS were cloned into the *Escherichia coli* expression vector pET28a(+). Constructs were transformed into *E. coli* BL21(λDE3). Transformed *E. coli* was grown at 37 °C in Terrific broth containing 50 μgml^{-1} kanamycin until an A_{600} value of 1.0 was reached. After induction with 0.1 mM isopropyl 1-thio-β-galactopyranoside, the cultures were grown for 6 h at 22 °C. Cells were harvested by centrifugation, resuspended in lysis buffer (50 mM Tris–HCl [pH 8.0], 500 mM NaCl, 20 mM imidazole [pH 8.0], 20 mM β-mercaptoethanol, 10% [v/v]

glycerol, 1% [v/v] Tween 20), and stirred at 4 °C for 1 h with lysozyme (0.5 mg/ml). After sonication and centrifugation, the supernatant was passed over a Ni^{2+}–NTA column and washed with 10 bed volumes of lysis buffer and 10 bed volumes of wash buffer (50 mM Tris–HCl [pH 8.0], 500 mM NaCl, 20 mM imidazole [pH 8.0], 20 mM β-mercaptoethanol, 10% [v/v] glycerol), and then the His-tagged protein was eluted with elution buffer (50 mM Tris–HCl [pH 8.0], 500 mM NaCl, 250 mM imidazole [pH 8.0], 20 mM β-mercaptoethanol, 10% [v/v] glycerol). Dialysis for 24 h at 4 °C against 25 mM Hepes, pH 7.5, 100 mM NaCl, and 1 mM dithiothreitol (DTT) was followed by concentration of protein solutions to greater than or equal to 10 $mg ml^{-1}$ by centrifugation using 30,000-molecular weight cut-off concentrators (Millipore). An equal volume of glycerol was added (50% [v/v] final concentration) for storage at −20 °C.

Single-vial assay for product analysis

The assay for recombinant sesquiterpene synthase activity was performed in 0.5-ml reaction volumes in assay buffer (50 mM Bis–Tris propane [pH 7.5], 20 mM $MgCl_2$) with 1.0 μM enzyme and variable concentrations of farnesyl diphosphate (ranging from 50 to 400 μM). Reactions were mixed at room temperature in 2-ml screw-top glass vials and overlaid with 0.5 ml of ethyl acetate, and the caps were affixed. After approximately 1 h incubation at room temperature, the hydrocarbon products were extracted by vigorous vortexing for 10–15 s, followed by GC–MS analysis (see below).

Steady-state kinetics assay

The steady-state kinetic assay for TEAS was performed in 0.5-ml reaction volumes in assay buffer (described above) with 0.1 μM enzyme and variable concentrations of farnesyl diphosphate (ranging from 2.5 to 160 μM). Reactions were mixed at room temperature (22 °C) in 2-ml screw-top glass vials and immediately overlaid with 0.5 ml of ethyl acetate containing 50 μM valencene as an internal standard, and the caps were affixed. After variable incubation times, reactions were terminated by vigorous vortexing (described above). Alternatively, reactions can be stopped by the addition of an equal volume of quenching solution (0.3 M ethylenediamine tetraacetic acid [EDTA], 0.6 M potassium hydroxide [KOH]) prior to overlaying the organic solvent. All assays were performed in triplicate with GC–MS data collected in the total ion monitoring (TIM) mode.

Product identification and quantification by GC–MS

Reaction products were analyzed using a Hewlett–Packard 6890 gas chromatograph (GC) coupled to a 5973 mass selective detector (MSD) outfitted with a 7683 series injector and autosampler and equipped with an HP-5MS capillary column (0.25 mm i.d. × 30 m with 0.25 μm film) (Agilent Technologies). The standard sampling depth places the needle position 3.6 mm above the vial bottom by default (considered position = 0 by the instrument). For these experiments, needle sampling depth was set to 8 mm (11.6 mm above the vial bottom), placing the needle in the middle of the organic layer (near the 750-μl level in the 2-ml glass vial). The GC was operated at a He flow rate of 2 $ml\,min^{-1}$, and the MSD was operated at 70 eV. Splitless injections (2 μl) were performed with an injector temperature of 250 °C. The GC was programmed with an initial oven temperature of 50 °C (5-min hold), which was then increased 10 $°C\,min^{-1}$ up to 180 °C (4-min hold), followed by a 100 $°C\,min^{-1}$ ramp until 240 °C (1-min hold). A solvent delay of 8.5 min was allowed prior to the acquisition of MS data. Product peaks were quantified by integration of peak areas using Enhanced Chemstation (version B.01.00, Agilent Technologies). The GC–MS instrument was calibrated with a valencene standard, which was also used as an internal standard for calculating reaction product quantities in the enzyme kinetic experiments. Corrected velocity data were fitted to the Michaelis–Menten equation using GraphPad Prism (version 4.00 for Windows, GraphPad Software). Product identification was performed using MassFinder 2.3 software (D. H. Hochmuth, D. Joulain, W. A. Konig, www.massfinder.com).

Extraction efficiency test

Sesquiterpene standards were diluted to a concentration of 100 mM in 100% ethanol. Various amounts of these stock solutions were added to buffered aqueous solutions identical to the enzyme assay conditions described above (50 mM Bis–Tris propane [pH 7.5], 20 mM $MgCl_2$) with vigorous vortexing. Aliquots (500 μl) were dispensed into glass vials and overlaid with 500 μl of organic solvent (pentane, diethyl ether, or ethyl acetate), and the caps were affixed. Vials were vortexed vigorously for 10–15 s, followed by GC–MS analysis as described above.

Results

The simplicity of design and ease of use of the vial assay are depicted in Fig. 1. Reaction conditions were formulated based on experimental objectives, and the assays were mixed in screw-top glass vials fitted with gas-tight Teflon septums. Organic solvent, typically ethyl acetate, was overlaid on the aqueous reaction mixture, and the caps were affixed (Fig. 1A). Incubation for variable times was followed by vigorous vortexing to partition hydrocarbon product into the organic phase. This

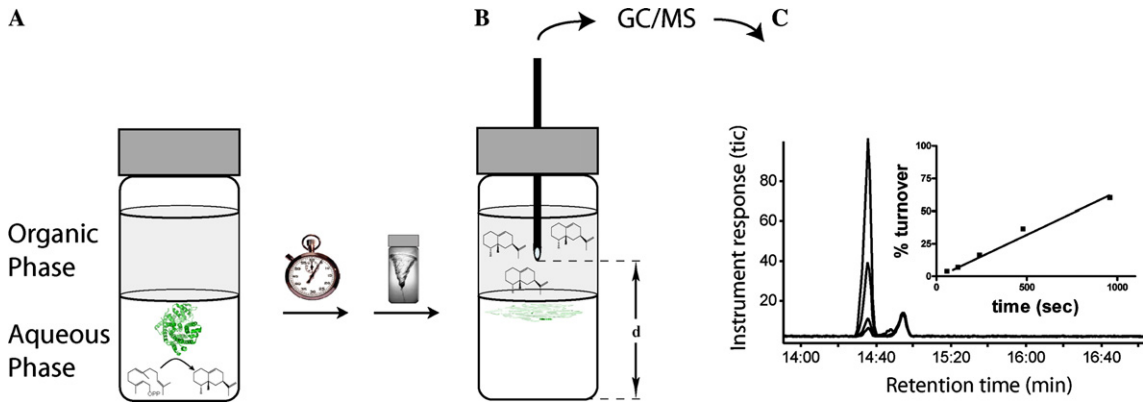

Fig. 1. Single-vial assay for sesquiterpene cyclase product analysis by GC–MS. (A) The cartoon depicts the typical vial system employed. (B) An injection needle is programmed to insert (d = distance) into the organic layer to withdraw a sample for GC–MS analysis. (C) A series of overlapped chromatograms with an inset plot (on the right) derived from vial assays for the quantitation of terpene hydrocarbons for kinetic analysis.

mixing step also quenched the reaction by denaturing the enzyme, which was often visible as a flocculent white precipitate at the organic/aqueous layer interface. With ethyl acetate as solvent, the aqueous and organic phases separate within minutes of vortexing, whereas hexane and pentane extractions produce a thick precipitate that requires brief centrifugation to separate. Adjustment of the needle sampling depth function on the instrument (for automated injection systems) allows one to selectively withdraw a portion of the organic phase for analysis of the extracted hydrocarbons by GC–MS (Fig. 1B). Thus, the reaction and extraction are performed in the same vial with no intervening sample manipulations. Quantification of products and internal standards through integration of peak areas can be used to determine relative amounts of product formed per unit time, which is a necessary step for subsequent kinetic analysis (Fig. 1C).

Quantification by GC–MS analysis

Instrument responses in GC–MS analyses, expressed as total ion current (TIC), reflect the number of ions affecting the detector at a given time. TIC can be measured by using total ion monitoring (TIM) mode, where all ions in the mass spectrum contribute to the measured response, or by using selected ion monitoring (SIM) mode to allow the quantification of specific ions in the mass spectrum. The scope of quantitative analysis by the vial assay was established by determining the detection limits and linear range of instrument responses in relation to the detection mode employed. Calibration curves were derived by quantification of at least five different concentrations of each of the sesquiterpene standards (–)-α-cedrene, valencene, and *trans*,*trans*-farnesol formulated in ethyl acetate. Relevant statistics are listed in Table 1. Linear regression analysis of the calibration data indicates a reasonable goodness of fit to a straight line with a correlation coefficient (r^2) greater than or equal to 0.99 for all standards used in either detection mode. The linear range of quantification, defined here as the lowest concentration detectable with a relative standard deviation (RSD) of 20%, was the most sensitive in SIM mode detection, extending down to low nanomolar concentrations of analyte (5 nM or 1 ng ml^{-1}) for (–)-α-cedrene. Despite the greater instrument response of TIM mode detection, the linear range extends only to 150 nM (~25 ng ml^{-1}) for the same standard due to a declining signal-to-noise ratio. SIM mode detection also afforded the lowest detection limits, defined here as the concentra-

Table 1
Calibration parameters and instrument limits[a]

Parameter[b]	Terpenes			
	(–)-α-Cedrene		Valencene	*trans*,*trans*-Farnesol
Mode	SIM[c]	TIM[d]	TIM	TIM
Slope (TIC/10^4)	2.71 ± 0.003	8.68 ± 0.15	8.77 ± 0.16	6.24 ± 0.11
Correlation coefficient r^2	0.999	0.998	0.999	0.999
Detection limits (μM)	0.001	0.13	0.107	3.3
Linear range (μM)	0.005–100	0.150–100	0.32–100	11.00–100

[a] Values were obtained using a Hewlett–Packard 6890 gas chromatographer as described in Materials and methods.
[b] Values for the slope of calibration plots are derived from measurements taken in the linear range of the instrument, which is defined here as the lowest concentration with an RSD of 20%.
[c] For SIM mode, the peak area of the dominant ion (base peak) in the mass spectrum of (–)-α-cedrene, *m*/*z* 119 was integrated for quantification.
[d] For TIM mode, the peak area for (–)-α-cedrene, including all ions, was integrated for quantitation.

tion that produces a chromatographic signal three times higher than the average background signal, extending as low as 1 nM analyte concentrations (0.2 ng ml^{-1}).

An important consideration for quantification of mixtures of terpene hydrocarbons is that instrument sensitivity varies depending on the analyte. This is particularly relevant to the characterization of terpene synthases that make multiple products with different degrees of cyclization and/or hydroxylation patterns. Comparison of the slopes and detection limits for the terpene standards used here illustrates this point (Table 1). Both (–)-α-cedrene and valencene calibration plots displayed similar slopes and the linear portions of the calibration curves extended into the nanomolar concentration ranges, whereas *trans*,*trans*-farnesol had a shallower slope and the effective range of quantification ended at low micromolar concentrations. Therefore, given the sensitivity differences among various terpenes, the common practice of expressing product abundances as a percentage of the total TIC of terpene products might not accurately reflect the absolute proportions of each product.

The organic phase used for extraction was found to influence subsequent quantification, as revealed through testing a few common solvents summarized in Table 2. The term "extraction efficiency," as used here, refers to the ability of a particular solvent to recover (extract) hydrocarbon from the aqueous phase using the vial assay extraction method. It is important to point out that in these experiments, substantial loss of analyte occurs during the dilution of hydrocarbon terpene standards into aqueous solutions due to volatilization. However, a single solution for a given terpene standard was extracted with each of the organic solvents to control for this, and so the variation in extraction efficiency between different solvents for the same terpene is strictly solvent dependent. Increasing solvent polarity, in going from pentane to ethyl acetate, generally resulted in greater extraction efficiencies with the exception of (–)-α-cedrene, for which extraction efficiency did not vary substantially from one solvent to another. The most striking effects were observed for *trans*,*trans*-farnesol, which was recovered nearly twice as efficiently with the most polar solvent, ethyl acetate, as compared with pentane and diethyl ether. This observation was not unexpected given the greater polarity of hydroxylated terpenes such as *trans*,*trans*-farnesol. Ethyl acetate proved to be the best overall solvent for the efficient extraction of terpene products in a single step and was employed for all subsequent studies.

In addition, instrument parameters used during analysis can affect the sensitivity and quantification of products. For instance, higher inlet temperatures volatilize more analyte and, hence, give rise to the highest instrument responses. However, care must be taken when using higher inlet temperatures because high temperatures can lead to thermal rearrangements of some terpenes, most notably the thermally induced cope rearrangement of germacrene A [17,18]. Moreover, oven heating programs and column matrices influence separations, sensitivity, and quantification but are not considered here.

Product identification

The efficacy of the vial assay for product identification was demonstrated using the sesquiterpene synthase, ADS. This enzyme was shown to produce eight minor products, three of which are hydroxylated, in addition to the expected major product amorpha-4,11-diene. A chromatogram from the GC–MS analysis of ADS reaction products is shown in Fig. 2. All previously reported ADS products were detected using the new assay method, and their mass spectra were sufficiently complete to match reference spectra in our library. The relative abundance of each product was expressed as a percentage of the total amount of terpene products found, as had been done in the previous report (Fig. 2, inset). In addition, five products not previously observed, accounting for approximately 2% of the total terpene

Table 2
Extraction efficiencies of sesquiterpene standards

Terpene	Retention time (min)[a]	Extraction efficiency[b]		
		Pentane	Diethyl ether	Ethyl acetate
(–)-α-Cedrene	14.7	31.7	24.3	30.6
Valencene	15.7	34.5	46.2	49.9
trans,*trans*-Farnesol	18.4	15.7	16.2	27.3

[a] Retention times of the standard compounds are relative and based on elution times under experimental conditions described in Materials and methods.

[b] Extraction efficiency is defined as the ratio of the measured product concentration (from GC–MS) to the starting concentration of analyte dissolved in aqueous solution (serially diluted from a known standard) multiplied by 100. The starting concentration differs from the actual concentration because analyte loss occurs due to volatilization during dilution of hydrocarbon standards into aqueous solution. However, a single solution for a given terpene standard was extracted with each of the different organic solvents to control for loss due to volatilization, and so the differences in extraction efficiencies among different solvents for the same terpene are strictly solvent dependent. Each value is the average of three or more measurements.

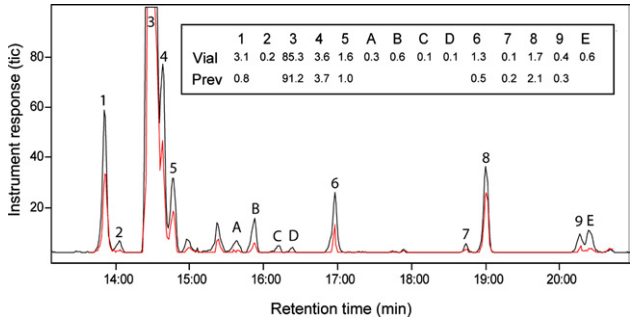

Fig. 2. GC–MS analysis via the vial assay of the sesquiterpene products of recombinant ADS. The overlay of two total ion chromatograms of sesquiterpene products generated from the vial assay performed with 50 μM FPP (in red) or 400 μM FPP (in black). The numbered peaks denote the sesquiterpenes assigned in the previous characterization of ADS (1) and correspond to (*E*)-β-farnesene (1), unknown (2), amorpha-4,11-diene (3), amorpha-4,7(11)-diene (4), γ-humulene (5), β-sesquiphellandrene (6), amorpha-4-en-11-ol (7) (tentative), amorpha-4-en-7-ol (8), and α-bisabolol (9) (tentative). Lettered peaks denote newly observed sesquiterpene products identified using the vial assay. The inset table lists the percentages of the total product that a given peak represents as derived from the vial assay (Vial) or previous (Prev) analysis. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

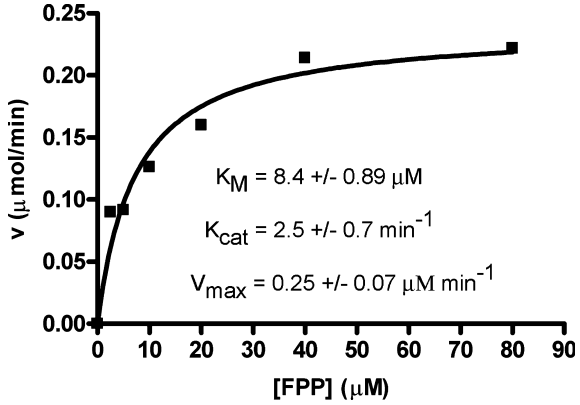

Fig. 3. Kinetic analysis of TEAS by the vial assay: Michaelis–Menten plot of data from the kinetic analysis of TEAS (as described in Material and methods) with determined kinetic parameters listed below the fitted curve.

products produced by ADS, were documented. Comparison of the mass spectra of these new compounds with a library of reference spectra suggests that several products originate from a bisabolyl cation, which is consistent with the previously proposed reaction mechanism (data not shown). The overlay of chromatograms from separate trials using either 50 or 400 μM FPP in the assay reveals a significant enhancement of minor product signals, with some only becoming visible under the latter condition. Given the defined limits of the GC–MS instrumentation as described above, and assuming complete conversion of FPP to product, detection of compounds constituting approximately 0.05–0.10% of the total products was possible, although only those that were 1% or greater (~0.5–1.0 μM) had mass fragmentation spectra of sufficient detail to reliably find matches to a library of sesquiterpene mass spectra.

Steady-state kinetics

Successful steady-state kinetic analysis entails the collection of measurements during the first 10% of substrate turnover, when the relationship between product formation and time is linear (Fig. 1C, inset). Construction of a Michaelis–Menten plot and derivation of kinetic parameters require a data set that encompasses substrate concentrations at or near the K_m of the enzyme. This typically ranges from 0.1 to 10.0 μM for most sesquiterpene synthases [2,10]. Given the linear range for quantification of sesquiterpenes outlined above, the vial assay is suitable for kinetic analysis of terpene synthases. This application was demonstrated using the sesquiterpene synthase, TEAS. Valencene was included in the organic phase as an internal standard and was used for quantification of 5-epi-aristolochene, the major reaction product of TEAS. Ideally, the internal standard and analyte should have similar retention times but with non-overlapping peaks. These compounds are diastereomers with retention times within 0.1 min of each other. A fitted curve ($r^2 = 0.952$) of TEAS activity versus FPP concentration gave rise to a typical hyperbolic saturation kinetics curve, as illustrated in Fig. 3. The derived kinetic parameters obtained from nonlinear regression analysis were comparable to previous values of k_{cat} and K_m obtained using a radioactive assay with [1-^{3}H]FPP. The k_{cat} values were essentially identical, with 2.5 min^{-1} measured using the GC–MS vial assay versus 2.9 min^{-1} reported previously using a radiometric assay [19]. The K_m determined here was 8.4 μM, which is reasonably close (within fourfold) of the previously reported value of 2.3 μM [19].

Discussion

The GC–MS has been used extensively to characterize the reaction products of terpene synthases, yet quantitative applications have remained underdeveloped to this point. This apparent deficiency has been due, in large part, to the time and labor required for sample preparation following enzyme assays. The vial assay developed here removes this barrier by offering a simple and standardized analytical method for terpene hydrocarbon product analysis. Typical detection limits achievable by GC–MS extend into the parts per billion range (0.1 ng ml^{-1} or 0.6 nM), as illustrated by the quantification of sex pheromones from *Anocentor nitens* [20]. Comparable detection limits were observed for the terpene standards used in this study, where (–)-α-cedrene was

detectable at concentrations as low as 1 nM in the SIM mode. This level of sensitivity, coupled with a standardized analytical method, fully enables the quantitative potential of GC–MS to be exploited for analysis of terpene synthases.

Validation of the vial assay as a quantitative method was demonstrated by steady-state kinetic analysis of TEAS. Termination of reactions by vortexing at various time points followed by quantification by GC–MS established the linear range of enzyme activity (Fig. 1C, inset), and a nonlinear fit of data to the Michaelis–Menten equation produced a hyperbolic kinetic saturation curve, as illustrated in Fig. 3. With previous methods, kinetic analysis of terpene synthases required the use of radioactivity to quantify the total extractable hydrocarbon reaction products. Many terpene synthases produce multiple products in addition to a dominant one, and there are exceptional synthases that have been documented to produce as many as 52 sesquiterpene olefins [7]. ADS, as characterized here, was shown to produce 14 products, 5 of which were not observed in previous studies. Furthermore, TEAS produced numerous products when incubated with FPP. The results of this improved assay, as applied to TEAS and its reaction mechanism, will be the topic of future publications currently in preparation. Importantly, the vial assay opens up the possibility of measuring the rates of formation of individual products that may arise from alternative reaction trajectories or premature terminations along the hydrocarbon cyclization pathway, thereby providing a more detailed picture of the electrophilic transformations catalyzed by terpene synthases. However, an important caveat to bear in mind for GC–MS quantification of mixtures of volatiles is that individual compounds may have different extraction efficiencies and instrument responses, thereby requiring the use of authentic standards for quantification of absolute analyte concentrations.

The short time frame and small scale associated with the vial assay make it amenable to high-throughput applications. For product identification, reactions were composed in 500-μl volumes with approximately 50 μg of purified protein. Kinetic experiments using the same reaction volumes were performed at 100-nM enzyme concentrations (~3 μg per reaction), which were sufficient for the complete conversion of substrate to products using the substrate concentrations described previously. With glass inserts or smaller vials, the reaction volume can be reduced to 50–75 μl. Elimination of intervening sample manipulations between reaction setup and product analysis not only reduces labor but also saves time, and so the rate-limiting step in the procedure becomes the GC–MS run and analysis. Even greater throughput can be achieved by coupling the single-vial assay to fast GC–MS methods [21]. In addition, the vial assay avoids the introduction of artifacts commonly found when using previously described approaches. Most notably, acid-catalyzed cyclization of germacrene A to α-seline and selina-4,11-diene results from passing pooled organic extracts over silica [22,23]. These products are absent in the GC traces of two germacrene A synthases, LTC1 and LTC2 [24], when analyzed by the vial assay under the lowest pH conditions at which the enzymes are active (pH 5) (data not shown).

In summary, the detection limits and the favorable linear range for terpene quantification associated with the vial assay described and analyzed in this study extends the utility of GC–MS analysis from sensitive product detection and identification to steady-state kinetic analysis. Most significant, this methodology removes the bottleneck imposed by previous methods and provides a standardized analytical procedure that increases the throughput of functional characterization of large libraries of mutant terpene cyclases possessing a range of diverse biosynthetic properties, including altered substrate and product specificity as well as altered kinetic properties (work in progress). Furthermore, consideration of the variables relevant to the detection and quantitation of terpene products should extend the applicability of this assay to the analysis of mono- and diterpene cyclases [25–27].

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