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RESEARCH ARTICLE

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The use of proton transfer reaction mass spectrometry for high throughput screening of terpene synthases

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Abstract

In this work, we introduce the application of proton transfer reaction mass spectrometry (PTR-MS) for the selection of improved terpene synthase mutants. In comparison with gas chromatography mass spectrometry (GC-MS)-based methods, PTR-MS could offer advantages by reduction of sample preparation steps and analysis time. The method we propose here allows for minimal sample preparation and analysis time and provides a promising platform for the high throughput screening (HTS) of large enzyme mutant libraries. To investigate the feasibility of a PTR-MS-based screening method, we employed a small library of *Callitropsis nootkatensis* valencene synthase (CnVS) mutants. Bacterial cultures expressing enzyme mutants were subjected to different growth formats, and headspace terpenes concentrations measured by PTR-Qi-ToF-MS were compared with GC-MS, to rank the activity of the enzyme mutants. For all cultivation formats, including 96 deep well plates, PTR-Qi-ToF-MS resulted in the same ranking of the enzyme variants, compared with the canonical format using 100 mL flasks and GC-MS analysis. This study provides a first basis for the application of rapid PTR-Qi-ToF-MS detection, in combination with multi-well formats, in HTS screening methods for the selection of highly productive terpene synthases.

KEYWORDS
high throughput screening, PTR-MS, sesquiterpene, valencene, VOCs

1 | INTRODUCTION

Many volatile organic compounds (VOCs) produced in plants are valuable compounds for the flavour and fragrance industry. In the last decade, great effort has been invested in the development of microbial platforms able to fermentatively produce plant-derived VOCs, resulting in the successful implementation of terpene synthases for the commercial production of terpenes.[1,2] To improve such microbial-based production systems and to select the best performing enzymes, reliable screening methods are needed. Currently,

the most widely used method is gas chromatography mass spectrometry (GC-MS). This technique is considered reliable and allows both qualitative and quantitative analysis of VOCs. However, as it requires multi-step sample preparation and long analysis times, it shows limitations in the throughput it can sustain.[3] Therefore, when screening for improved enzyme mutants, GC-MS can only be employed as a medium-throughput screening method.

Proton transfer reaction mass spectrometry (PTR-MS) is a widely used technique for the analysis of VOCs.[4] This technique has been developed to have high sensitivity, allowing to detect VOCs in complex mixtures in the range of parts per trillion. The PTR-MS uses positively ionized small molecules, typically H_3O^+, as proton donors for VOC ionization.[5] Several types of mass spectrometers can be employed for the detection of the ionized VOCs, as mass-to-charge ratio (m/z),[6] such as a time-of-flight mass spectrometer (ToF-MS).[7] The ToF-MS guarantees increased dynamic range and sensitivity in comparison with other PTR-MS formats and reaches higher performance when coupled with a quadrupole ion guide (Qi).[8,9]

PTR-MS is used for a variety of different applications, from air quality control monitoring[10] to food technology[11] and medical research.[12] The most compelling feature offered by the use of PTR-MS is the possibility to detect VOCs in real time. This allows a reduction in sample preparation and analysis times, when compared with other analytical techniques, thereby introducing a potential for high throughput screening (HTS) of a large number of samples. Several studies report the application of PTR-MS for monitoring the shelf life and storage effects on milk products[13,14] and their volatile profiling for quality control,[15] as well as monitoring the presence of different bacterial contaminants.[16]

In plants, PTR-Qi-ToF-MS has been employed to analyse the emission of isoprenoids, with a throughput of over 700 samples per day.[17] This work revealed that PTR-MS analysis allows a much faster throughput of samples than GC-MS-based techniques.[18,19] Sesquiterpenes belong to the isoprenoid family and are synthesized by plants as part of their essential oils, playing an important role in plant communication.[20] Being VOCs, sesquiterpenes can be detected by PTR-MS in different types of samples. A reliable quantification of total sesquiterpene signal can be obtained by monitoring emissions of m/z 205 from plant samples,[21] whereas real-time measurements of VOCs over time allow the non-invasive analysis of metabolites emissions.[22] Among the sesquiterpenes, valencene has been of interest for the flavour and fragrance industry, being a component of citrus essential oils and the precursor of nootkatone, the main grapefruit flavour.[23]

In this work, we propose the novel application of PTR-Qi-ToF-MS for the screening of sesquiterpene synthase mutants, using a previously tested valencene synthase mutant library, including variants showing different efficiency. By comparing these results with those obtained by using GC-MS analysis, we are able to demonstrate that PTR-Qi-ToF-MS offers a promising alternative as an HTS method to identify improved sesquiterpene synthase mutants.

2 | MATERIALS AND METHODS

2.1 | Bacterial strains

An *Escherichia coli* strain derived from BL21(DE3), harbouring a plasmid expressing all genes necessary for the synthesis of farnesyl diphosphate (FDP) (pBbE5k-MevT (CO)-MBIS (CO)),[24] was used for the production of valencene. *C. nootkatensis* valencene synthase (CnVS)[25] was chosen as a target for this experiment, together with a small library of single mutants (plasmids were kindly provided by Isobionics BV) (Table 1). Synthases were expressed using the inducible plasmid pACYCDuet-1, as previously described.[24]

2.2 | Cultivation conditions

For the experimental setup, different conditions were chosen, consistently with the analysis technique to apply.

2.2.1 | Cultivation conditions for GC-MS analysis

Fermentations were performed as previously described.[24] In short, pre-cultures were grown in 5 mL LB medium supplemented with 50 µg/mL kanamycin and 50 µg/mL chloramphenicol in 50 mL Greiner tubes. Subsequently, the pre-cultures were diluted to an OD600 of 0.150 in 20 mL 2xYT medium with antibiotics in 100 mL glass flasks and incubated at 37°C 250 rpm until an OD600 of 0.4–0.5 was reached. Then, 1 mM IPTG and 10% v/v n-dodecane 99% (Alfa Aesar) were added to the fermentations, following a 24 h incubation at 28°C 250 rpm. The dodecane layer was recovered for GC-MS analysis by centrifugation at 4600 rpm for 15 min. All selected CnVS mutants were grown in duplicates for GC-MS analysis.

2.2.2 | Cultivation conditions for PTR-Qi-ToF-MS analysis

Fermentations were performed using alternatively 30 mL of 2xYT medium with antibiotics in 250-mL bluecap glass bottles, or 20 mL of 2xYT medium with antibiotics in 100 mL bluecap glass bottles (Greiner). The same growth conditions were applied as described for the GC-MS analysis. As no extraction of the sesquiterpenes in organic phase is necessary for PTR-Qi-ToF-MS analysis, no dodecane layer was added to the cultures.

In a smaller scale setup, reusable 24-square deep-well (dw) polypropylene plates (17 × 17 mm, depth 40 mm, total volume 10 mL, Applikon Biotechnology, Netherlands) and single-use 96-square dw polypropylene plates (8 × 8 mm, depth 40 mm, total volume 2 mL, VWR Netherlands) were used. 800 µL and 200 µL of 2xYT medium with antibiotics were inoculated for the 24 dw plates and the 96 dw plates, respectively. Cells were inoculated at an

TABLE 1 Library of selected sesquiterpene synthases, performances are reported for fermentations.

CnVS library	Performance	GC-MS quantification (mg valencene/L)[a]	Reference
CnVS	Wild Type	69.4 ± 5.2	Beekwilder et al.[25]
F300Y	Lower production	57.5 ± 0.4	Isobionics BV
L444F	Loss of function	ND[b]	Isobionics BV
L566S	Higher production	90.9 ± 2.1	Isobionics BV
pACYCDuet-1	Empty plasmid	ND[b]	Laboratory stock

Note: The column on the right reports the valencene quantification obtained via GC-MS analysis.
[a]Quantification performed on n-dodecane extracted from in vivo fermentations using 20-mL medium in 100-mL flasks.
[b]Not detectable (ND).

OD600 of 0.1, and 1 mM IPTG was immediately added. Twenty-four dw plates were covered using Air-O-seal hydrophobic gas permeable adhesive seals (Bioké, Netherlands), whereas 96 dw plates were covered by silicon Axygen AxyMat (96 Square Well Sealing Mat for Deep Well Plates, VWR, Netherlands). Plates were incubated using the MicroFlask shaker clamp system (Applikon Biotechnology, Netherlands) at 28°C and 300 rpm for 24 h.

The OD600 was determined for each sample, after the PTR-ToF-Qi-MS measurement. Measured growth in flasks was used as a benchmark, and cultures were grown in duplicate for each experiment. The same pre-culture of each strain was used for all cultivation conditions. Two plates per type were measured as technical replicates. Twenty-four dw plates setup was designed to contain three biological replicates of each strain, whereas 96 dw plates setup was designed to contain five biological replicates of each strain.

2.3 | GC-MS measurements and data analysis

GC-MS analysis was performed as described before.[24] In brief, a 7980A GC system combined with a 5975C inert MSD detector (70 eV) (Agilent Technologies, Netherlands) was used. The system is equipped with a 7683 auto-sampler and injector and a Phenomenex Zebron ZB-5-ms column of 30 m length × 0.25 mm internal diameter and 0.25 µm stationary phase, with a Guardian precolumn (5 m). For each sample, 1 µL of the sample was injected. The injection chamber was at 250°C, the injection was splitless, and the ZB5 column was maintained at 55°C for 2 min after which a gradient of 10°C per minute was started, until 300°C. Peaks were detected in chromatograms of the total ion count. Valencene was identified and quantified by comparing the sample peak with a valencene standard (80% purity, Isobionics, Netherlands).

Average and standard deviation of replicates was calculated using Microsoft Excel. GC-MS results were used as the benchmark for the validation of PTR-Qi-ToF-MS measurements.

2.4 | PTR-Qi-ToF-MS measurements

For the measurements of bottles and plates, a PTR-Qi-ToF-MS (Ionicon Analytik GmbH, Innsbruck, Austria) operating in V mode (standard configuration) was used to measure the headspace of the samples. The following ionization conditions were set in the drift tube [900 V, 60°C and 3.80 mBar corresponding to an E/N value of 134 Townsend (1 Td = 10^{-21}Vm^2)]. The reagent ion used in this system is H_3O^+. Two sampling approaches were taken for the different sample typology The mass resolution (m/Δm) was higher than 3800. A polyetheretherketone (PEEK) capillary tube (inner diameter, 0.50 mm), heated at 60°C, was used as an inlet. The inlet tube was connected to a standard ISO polypropylene screw cap GL45 (max temperature 140°C), supplemented with two fitting valves. The second valve is necessary for the air flow in entrance. The flow rate was of 52 sccm (cm^3/min at standard temperature 273 K, pressure 1 atm), with an acquisition rate of one spectrum per second (*m/z* range: 20–510). Assuming the headspace volume is 220 mL (250-mL bottle), 80 mL (100 mL bottle), 9.2 mL (24 dw plate) and 1.8 mL (96 dw plate), this would mean that the whole headspace is flown through the PTR-Qi-ToF-MS in 190, 69, 8 and 1.56 s, respectively. To standardize the measurement setup, discrete 60-s measurements were performed for both 250 and 100 mL bottles. Before each measurement, the bottles were heated at 30°C for 30 min to allow gas equilibration. After 10 s of sampling laboratory air, each sample was measured for 40 s. For the measurements of 24 and 96 dw plates, the probe was attached to a sterile Henke-Ject® 0.90 × 25 mm needle (Henke Sass Wolf, Germany). Another needle of the same type was used to guarantee the airflow in the wells during the measurements. Plates were not heated beforehand, to avoid variation due to cooling down throughout the measurement. In this case, a continuous measurement was performed, and samples in different wells were measured without interruption. Each sample was measured for 10 s, with a recovery time (airflow) of 40–60 s between each sample to avoid memory effects. All measurements were performed manually. All *m/z* values in this study are reported as integers mass accuracy approximated for consistency. The parent ion for sesquiterpenes $(C_{15}H_{24})^+$ is *m/z* 205.195[26] and is indicated in this work as *m/z* 205.

2.5 | PTR-Qi-ToF-MS data processing and analysis

All the PTR-Qi-ToF-MS data were processed through the PTR-MS Viewer software v.3.4.2.1 (Ionicon Analytik GmbH, Austria). Firstly, an internal mass axis calibration was performed by using the following calibration mass peaks: 21.0226 (H_3O^+), 29.9974 (NO^+), 59.0497

($C_3H_6OH^+$) and 203.9430 (fragment of 1,3-diodobenzene used as internal gas standard). The final mass accuracy of the processing was sufficient for determining the sum formula of volatile compounds (≈0.001), and therefore, the experimental *m/z* values are reported up to the third decimal digits. Then, a mass peak integration was conducted to extract peak intensities in counts per second (CPS) and in normalized CPS (NCPS) using the formula:

$$NCPS = 10^6 \times \left([MH^+]/\left(\left([H_3O^+]/0.2\right) + [cluster\,H_2O + H^+]\right)\right)$$

where [MH^+]: *m/z* 205 cps; [H_3O^+]: *m/z* 21 cps; [cluster H_2O H^+]: *m/z* 37 cps.

For bottles, an average of the NCPS from second 15 to second 45 after insertion of the probe was calculated, whereas for plates, an average of the NCPS from the moment of insertion of the probe start until 30 s after insertion of the probe was used. The baseline was calculated as an average of all the blank samples (culture medium) and subtracted from the average NCPS. For statistical analysis, IBM SPSS was used. To assess homogeneity of variance, Levene's test for equality of variances of the one-way ANOVA analysis was performed. For *p*-values higher than 0.05, the assumption of homogeneity of variance is met and the one-way ANOVA was performed, using Tuckey and Scheffe post hoc tests. For *p*-values lower than 0.05, the assumption of homogeneity of variance is violated and a nonparametric Kruskal–Wallis test was used to conduct the analysis. *T*-test assuming equal variance was also performed.

3 | RESULTS

3.1 | Mutants of CnVS show different enzyme activity in vivo and in vitro

CnVS has previously been shown to produce mainly valencene, with around 10% germacrene A, which is observed as its heat-induced rearrangement product, β-elemene[25] (Figure 1A). A small library, consisting of the empty vector (pACYCDuet-1), CnVS and three CnVS mutants, was tested. The selected mutants carried substitutions in the following amino acid positions: 300 (F300Y), 444 (L444F) and 566 (L566F) (Table 1). These enzymes were expressed in *E. coli* BL21DE strain, and the production of sesquiterpenes was assessed using a biphasic fermentation using 100 mL flasks. Cultures were grown in a 20 mL medium, with an organic phase of n-dodecane, to trap the produced sesquiterpenes. We observed that mutant L566S showed a higher production of valencene compared with CnVS, whereas, as expected, no valencene was detected for L444F (Figure 1A,B). Finally, variant F300Y performed less well than CnVS.

3.2 | PTR-Qi-ToF-MS analysis of cultures in bottles

The CnVS mutants were subsequently grown in 100- and 250 mL glass bottles, with 20 or 30 mL culture, respectively. In the bottles, no n-dodecane overlay was added because no extraction is necessary for the analysis of the produced sesquiterpene in the culture headspace by PTR-Qi-ToF-MS. Moreover, a preliminary experiment suggested that the presence of 10% (v/v) n-dodecane interferes with the detection of sesquiterpenes as the high concentration of n-dodecane, being a VOC itself, saturated the detector and caused strong ion suppression. In parallel, starting from the same pre-cultures, flasks with 20 mL culture and n-dodecane layer were used as benchmark for growth and production of sesquiterpenes, measured with GC-MS. The growth in flasks was comparable with the growth in bottles, with no major difference for different bottle sizes, as shown in Figure 2A. After 24 h fermentation, the headspace of the bottles was analysed with PTR-Qi-ToF-MS using 60 s discrete measurements (Figure 2B).

For both bottle sizes, the PTR-Qi-ToF-MS signals of the different CnVS mutants were consistent with the GS-MS-based quantifications of the flask cultivations. In all three setups, no signal was detected for

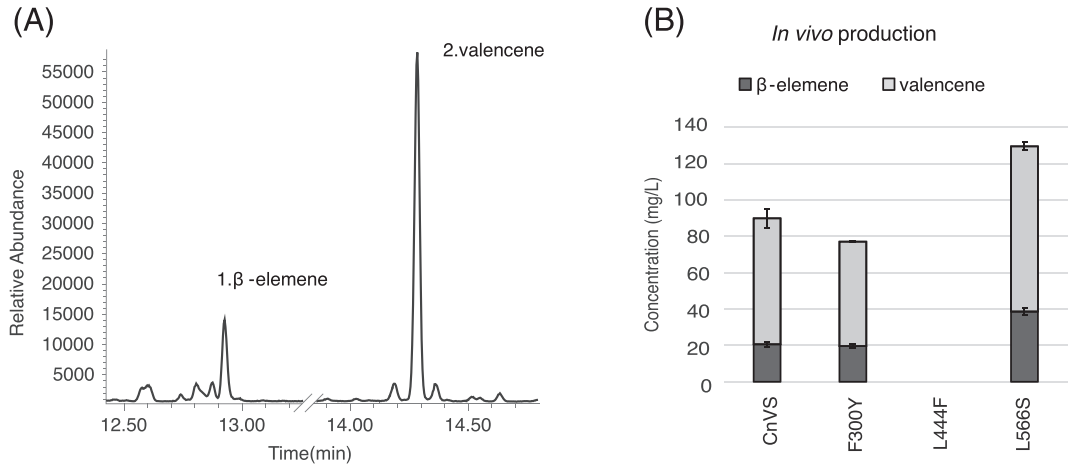

FIGURE 1 Characterization of valencene synthase from *Callitropsis nootkatensis* (CnVS) mutants. (A) Example GC-MS chromatogram of sesquiterpene produced using wild type CnVS. Peaks for (1) β-elemene (heatinduced rearrangement product of germacrene A) and (2) valencene are reported. (B) Fermentative production of sesquiterpenes by CnVS mutants. The sesquiterpenes were quantified via GC-MS analysis.

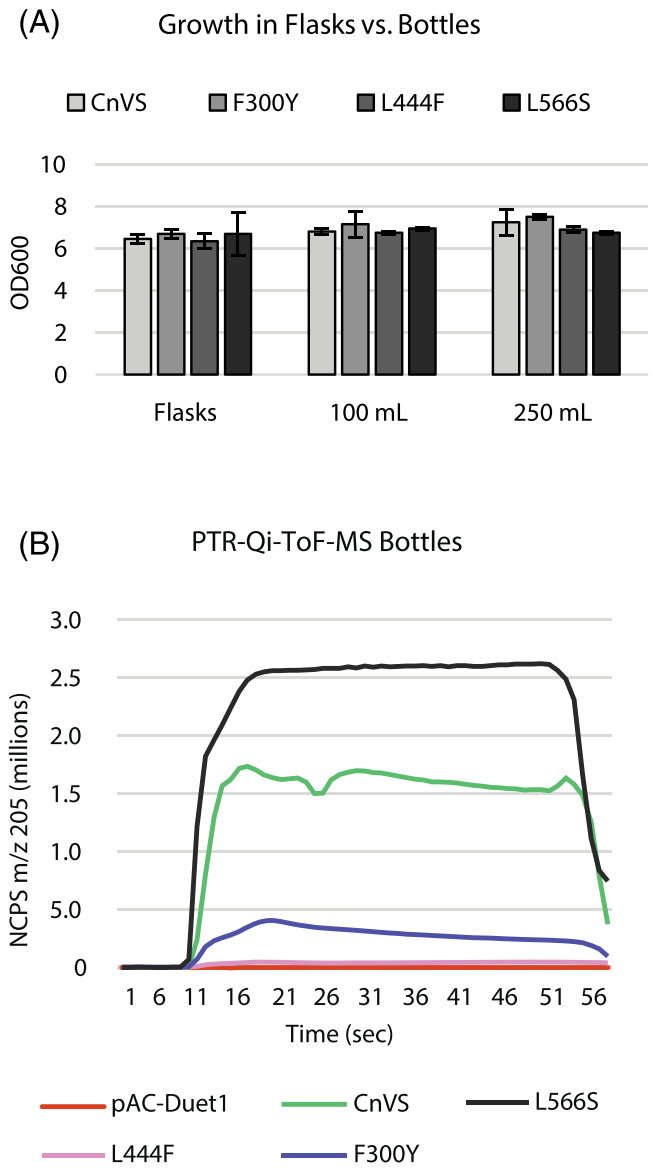

FIGURE 2 (A) Growth comparison of CnVS mutants between cultures in flasks and in bottles. OD600 was measured after 24 h. Growth in flasks and in bottles showed no significant difference. (B) Summary graph of PTR-Qi-ToF-MS analysis of cultures grown in bottles. The graph reports the measurement of the headspace of cultures grown in 30-mL medium. L566S shows the highest signal. Empty plasmid pACYCDuet-1 is abbreviated as pAC-Duet1.

sesquiterpenes in L444F, as expected, whereas F330Y, CnVS and L566S showed increasing sesquiterpenes signals, respectively (Figure 3A,B,C). To further corroborate the similarity of the analytical methods, a correlation analysis was performed (Figure 3D,E), showing a good correlation between the flasks (R^2 > 0.95) and both bottle types. These experiments demonstrate the applicability of PTR-Qi-ToF-MS as a screening method for variations in terpene synthase efficiency, showing a good correlation with GC-MS, but with the advantage of a much shorter measurement time. A larger difference between the ratio L566S/F300Y was observed in bottles compared with flasks, especially for the 250 mL bottle setup (Table 2). This result may indicate a different sensitivity of the methods at lower concentrations, or a difference due to the different headspace in the 250 mL bottles. Mean, standard deviation and standard error were calculated (Table S1).

3.3 | PTR-Qi-ToF-MS analysis of cultures in deep-well plates

To assess the suitability of the PTR-Qi-ToF-MS as an HTS method for larger mutant libraries, a smaller scale cultivation setup was tested. The same strains used to perform measurements of valencene production with PTR-Qi-ToF-MS in bottles were grown in 24 dw plates. This allowed to scale down the volumes, using 800 µL of the medium. For each CnVS mutant, three replicates were grown in parallel, and the experiment was repeated twice. For this setup, a continuous measurement approach was used, in which each well was measured for 10 s and a recovery time varying between 30 and 90 s was taken between measurements, to eliminate any possible memory effect (Figure 4A). As benchmark, cultures were also grown in flasks with n-dodecane overlay; the valencene production was analysed using GC-MS and quantified using a valencene standard (Figure 4C). With the 24 dw plates setup, clear differences were observed between the cultures measured with PTR-Qi-ToF-MS (Figure 4D and Table S1). Consistently with the previous larger volume experiments, when comparing cultures grown in flasks and in bottles (Figure 3A,B,C), L566S showed on average higher valencene production than CnVS. Similarly, the signal for valencene produced by F300Y was consistently lower than CnVS, and valencene production by L444F was hardly detectable (Figure 4D). After measuring the headspace of the 24 dw plates, bacterial growth was measured as OD600, showing no major differences from the cultures grown in flasks (Figure 4F).

A further miniaturization of the cultivation was introduced by growing the strains in a 96 dw plate format. In this case, the cultures were grown in 200 µL medium. The same PTR-Qi-ToF-MS analysis time and setup as for the 24 dw plates were used (Figure 4B), and experiments were repeated twice, on different days. Each strain was cultivated in five replicates per plate. In this experiment, the difference between valencene produced by L566S and CnVS was less pronounced (Figure 4E) and did not qualify as significant in the ANOVA analysis, although the mean value for L566S was higher than the value for CnVS. OD_{600} was measured for 96 dw plates to assess bacterial growth. No significant differences were detected between the different mutants and results comparable with the growth obtained in flasks and in 24 dw plates (Figure 4F). Twenty-four and 96 dw plates were grown in parallel with the flasks. It must be noted that the GC-MS results for the flask cultivations ran in parallel also showed a less pronounced difference between the production of valencene by CnVS and L566S (Figure 4C) when compared with the previous experiment (Figure 3A). Mean, standard deviation and standard error for 24 and 96 dw plates were calculated (Table S1). Interestingly, the ratio of sesquiterpene produced in L566S compared CnVS to is similar in flasks and in plates, whereas much higher ratios are observed in L566S/

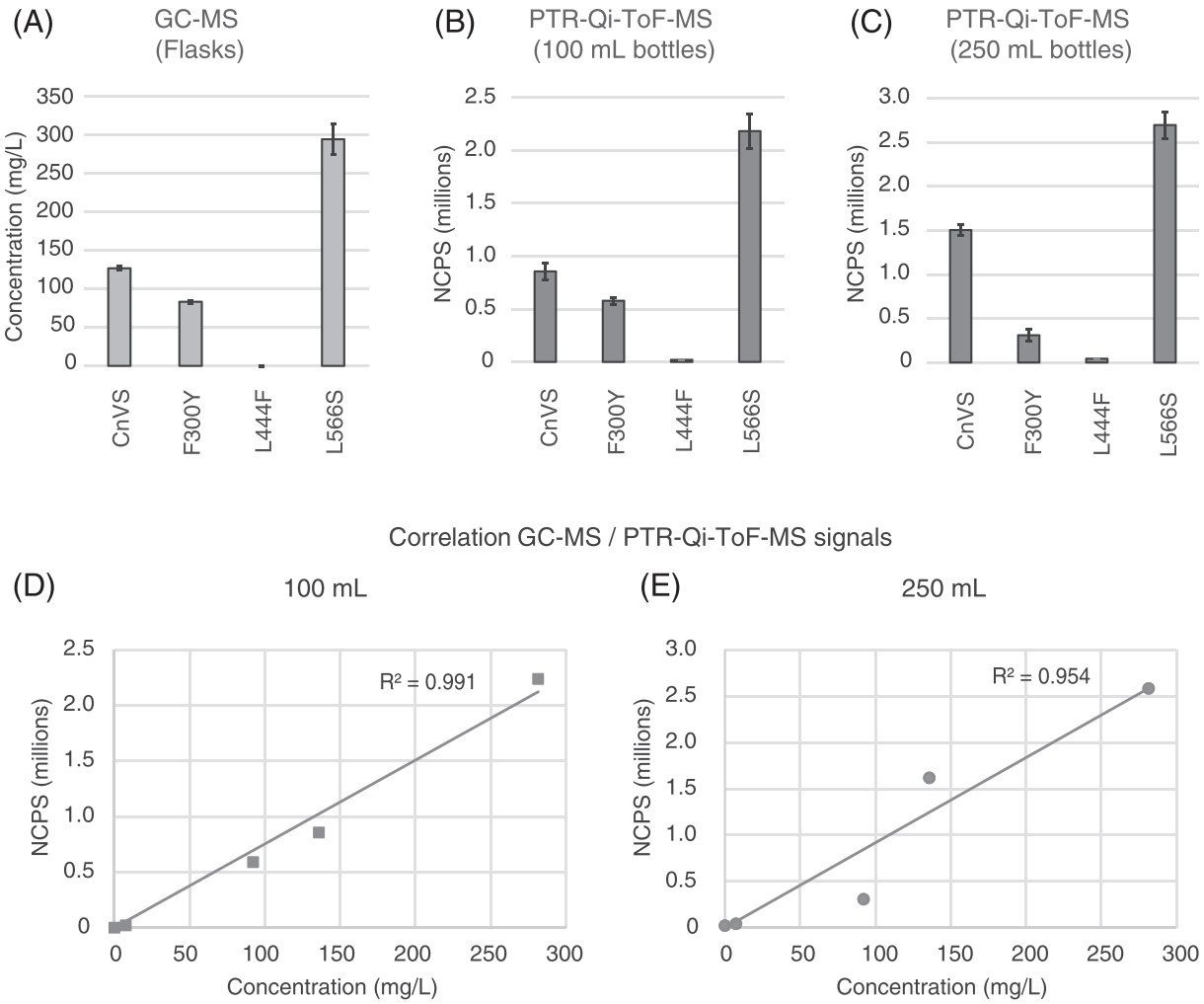

FIGURE 3 Comparison of GC-MS and PTR-Qi-ToF-MS analysis. (A) GC-MS analysis of sesquiterpene produced in flasks using n-dodecane as overlay. The graph reports the total sesquiterpenes as concentration (mg/L). (B–C) PTR-Qi-ToF-MS analysis of fermentation in (B) 100 mL and (C) 250 mL glass bottles. (D–E) Correlation between mg/L (*x*-axis) and NCPS in 100 mL bottles (D) and 250 mL bottles (E).

TABLE 2 Calculated ratio for sesquiterpene production in flasks (measured with GC-MS), and bottles (measured with PTR-Qi-ToF-MS).

	GC-MS Flasks	PTR-Qi-ToF-MS Bottles	
Ratios	**100 mL**	**100 mL**	**250 mL**
L566S/CnVS	2.2	2.6	1.8
L566S/F300Y	3.3	3.8	8.7
CnVS/F300Y	1.5	1.5	4.9

Note: Ratios were calculated using the mean values obtained for each condition. Mean values, standard deviation and standard error are reported in Table S1.

F300Y and CnVS/F300Y (Table 3), as observed in the previous experiment. These differences may account for a different sensitivity of mutant F300Y to the different growth conditions and the difference in the relative headspace measured.

When comparing the results obtained for 24 and 96 dw plates (Figure 4G), we observe a very good correlation (R^2 > 0.97). This supports the robustness of the miniaturization system and indicates that the 96 dw plate can be employed for HTS.

4 | DISCUSSION

In our study, we present a fast and reliable method for HTS of terpene synthase mutant libraries. So far, HTS of terpene-producing microbial strains has mainly been developed using colorimetric methods. Reactive dyes such as Purpald[27,28] or malachite green[29,30] have been employed for screening of terpene synthase activity in vitro.[27,29] However, caution needs to be used when relating the activity of synthases in vitro with the in vivo performance of strains expressing mutants of the synthases. The different conditions of the in vitro environment may not be representative of the in vivo environment, as different physical and chemical conditions, such as pH, salinity and

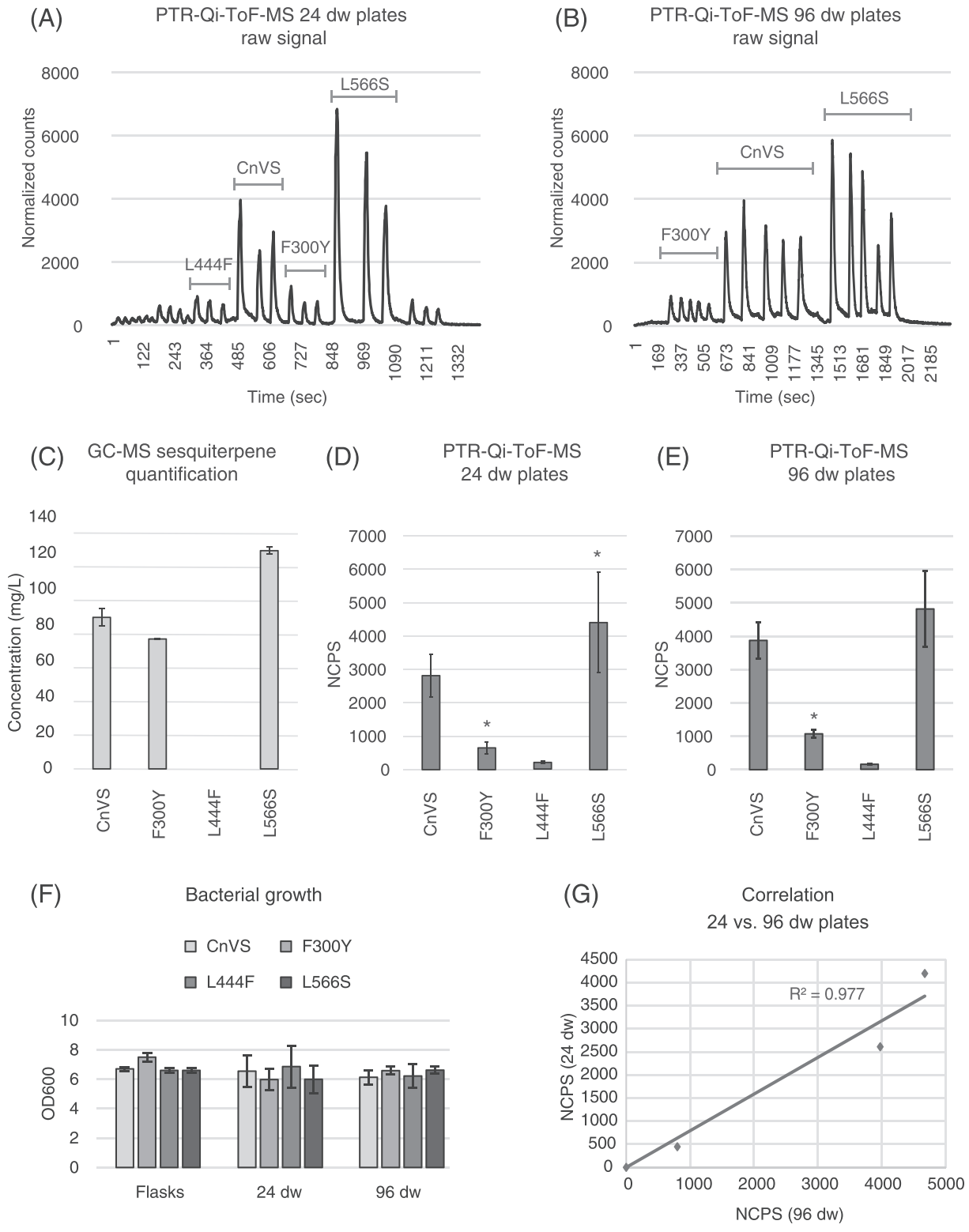

FIGURE 4 Raw signal obtained for continuous measurements of (A) 24 dw and (B) 96 dw plates measured with PTR-Qi-ToF-MS. Replicates can be recognized in the varying peak heights. (C) Valencene produced in flasks was quantified using GC-MS. Mean values of NCPS were calculated for (D) 24 dw and (E) 96 dw plates. The asterisk indicates a significant difference compared with the wild-type CnVS ($p < 0.05$). F300Y and L566S show significant difference compared with CnVS for 24 dw plates (D), while for 96 dw plates, only F300Y shows significant difference compared with CnVS (E). (F) Comparison of bacterial growth in flasks, 24 and 96 dw plates. The growth was comparable among the three different setups. (G) Correlation between signals (NCPS) obtained with PTR-Qi-ToF-MS for 24 and 96 dw plates.

TABLE 3 Calculated ratio for sesquiterpene production in flasks (measured with GC-MS) and dw plates (measured with PTR-Qi-ToF-MS).

	GC-MS Flasks	PTR-Qi-ToF-MS Plates			
	100 mL	24 dw		96 dw	
	Ratio	Ratio	T-test	Ratio	T-test
L566S/CnVS	1.4	1.6	0.1	1.2	0.2
L566S/F300Y	1.7	6.7	2.9E-03	4.5	1.8E-04
CnVS/F300Y	1.2	4.3	2.7E-05	3.6	8.3E-06

Note: Ratios were calculated using the mean values obtained for each condition. Mean values, standard deviation and standard error and are reported in Table S1. *T*-test was included for 24 and 96 dw plates.

availability of the substrate, might hinder or enhance the activity of the enzyme without a correspondence in the in vivo system.

Colorimetric approaches for HTS for in vivo activity have been developed, such as the use of Nile Red to select *E. coli* strains that accumulated higher concentrations of terpenes in their intracellular space.[31] Another qualitative method reported for the screening of monoterpene synthases (limonene) relied on the use of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and its chromatic toning in organic phase because of the mild antioxidant activity of some monoterpenes.[32] These methods, although being faster than GC-MS analysis, present a number of limitations related to the selectivity of the staining agent and the subsequent detection method. For instance, the detection of Nile Red is subject to variation due to background noise related to the staining of lipids. DPPH is known to react with other antioxidant compounds and has been widely applied as an in vitro assay for antioxidant activity.[33] This lack of specificity decreases the accuracy of the results, as small variations in the presence of other, stronger, antioxidant compounds could interfere with the detection of terpenes. Furthermore, both these methods are indirect and require an extra step of sample preparation that reduces the throughput of the screening.

In the current work, we evaluate real-time detection of sesquiterpenes in the headspace of the cultures as a tool for HTS. The PTR-Qi-ToF-MS methodology provided results comparable with the classical analytical method based on GC-MS when testing a mini library of valencene synthase mutants in increasingly miniaturized setups. Indeed, measurements performed by comparing cultures grown in bottles and flasks show that sesquiterpenes present in the headspace measured by PTR-Qi-ToF-MS correlate well (R^2 > 0.95) with sesquiterpenes present in the dodecane layer measured by GC-MS (Figure 3D,E). Because the physiological conditions in these two tests are comparable, this experiment indicated that the PTR-Qi-ToF-MS analysis may replace the time and labour-intensive GC-MS.

To further address the robustness of the PTR-Qi-ToF-MS method and its applicability as HTS method, we tested the same library in miniaturized cultivation setups. The variation in valencene detected between replicates observed when analysing dw plates was highest for the 96 dw plate setup (Table S1). This variation might be caused by the smaller culture volume and the potentially more dramatic impact of the biomass growth in correlation with the production. In fact, it must be noted that a different ratio of volume/culture was used for 24 and 96 dw plates. In particular, a headspace of 9.2 mL was measured for 24 dw plates, having a culture volume of 800 µL, whereas a headspace of 1.8 mL was measured for 96 dw plates, with a culture volume of 200 µL (Figure S1). Therefore, the headspace for 24 dw plates represented 92% of the total volume, whereas for 96 dw plates, it was 90%.

We showed that by using the standard PTR-Qi-ToF-MS flow (52 sccm) and 10 s per measurement, with a 40 s recovery time, a good separation of the peaks is possible for 24 and 96 dw plates, allowing to distinguish between the different samples (Figure 4A–B). However, to further improve the performance, a higher measuring frequency could be considered for the plates. The variation of frequency can have an impact on the performance of PTR-MS measurements. As the frequency of the ionization source determines the rate at which the protons are transferred to the analyte molecules, changing the frequency can affect the sensitivity and accuracy of the measurements. At higher frequencies, the rate of proton transfer is faster, resulting in higher ionization efficiencies and greater sensitivity. However, caution should be applied, as increasing the frequency also increases the likelihood of ion-molecule reactions, which can lead to the formation of unwanted by-products and interfere with the detection of the analyte ions.[4] With the described setup, we showed that under each cultivation condition tested, the ranking of the mutants was the same, indicating a high sensitivity of the PTR-Qi-ToF-MS for detecting changes in terpene production, even in the smallest scale of the 96 dw plates. Indeed, we observed that mutant L566S consistently produced more valencene than CnVS (Figures 3B–C and 4D–E), whereas mutant L444F was the least efficient. In all cultivation conditions tested, F300Y was less productive than CnVS. However, it should be noted that this mutant showed differences in performance in the different experimental repetitions using the standard flask cultivation conditions. This suggests that this mutant might be more sensitive to small fluctuations in environmental conditions. Similarly, in both 24 and 96 dw plates, the production of valencene by F300Y was significantly lower than that of CnVS (Figures 4D–E). However, a good correlation between the production in 24 and 96 dw plates is shown in Figure 4G, indicating that the further miniaturization has only a limited impact on production and detection. Although the ratio between the production of valencene in CnVS and F300Y was much higher in 24 and 96 dw plates compared with flasks (Table 3), CnVS consistently outperformed F300Y. This result is in line with the application of PTR-Qi-ToF-MS as HTS method, where the focus would uniquely be on the enzymes showing higher productivity compared with the wild type. Noticeably, any VOC produced by terpene synthases in a bacterial system can be detected using PTR-MS, extending the applicability of this method to a wide variety of compounds.

Although the results obtained by using the dw plates are consistent with the GC-MS results obtained in the flasks from the same preculture, a smaller cultivation volume could influence the difference in terpene accumulated within the 24 h of cultivation. To our knowledge, the impact of a relatively small headspace on terpene

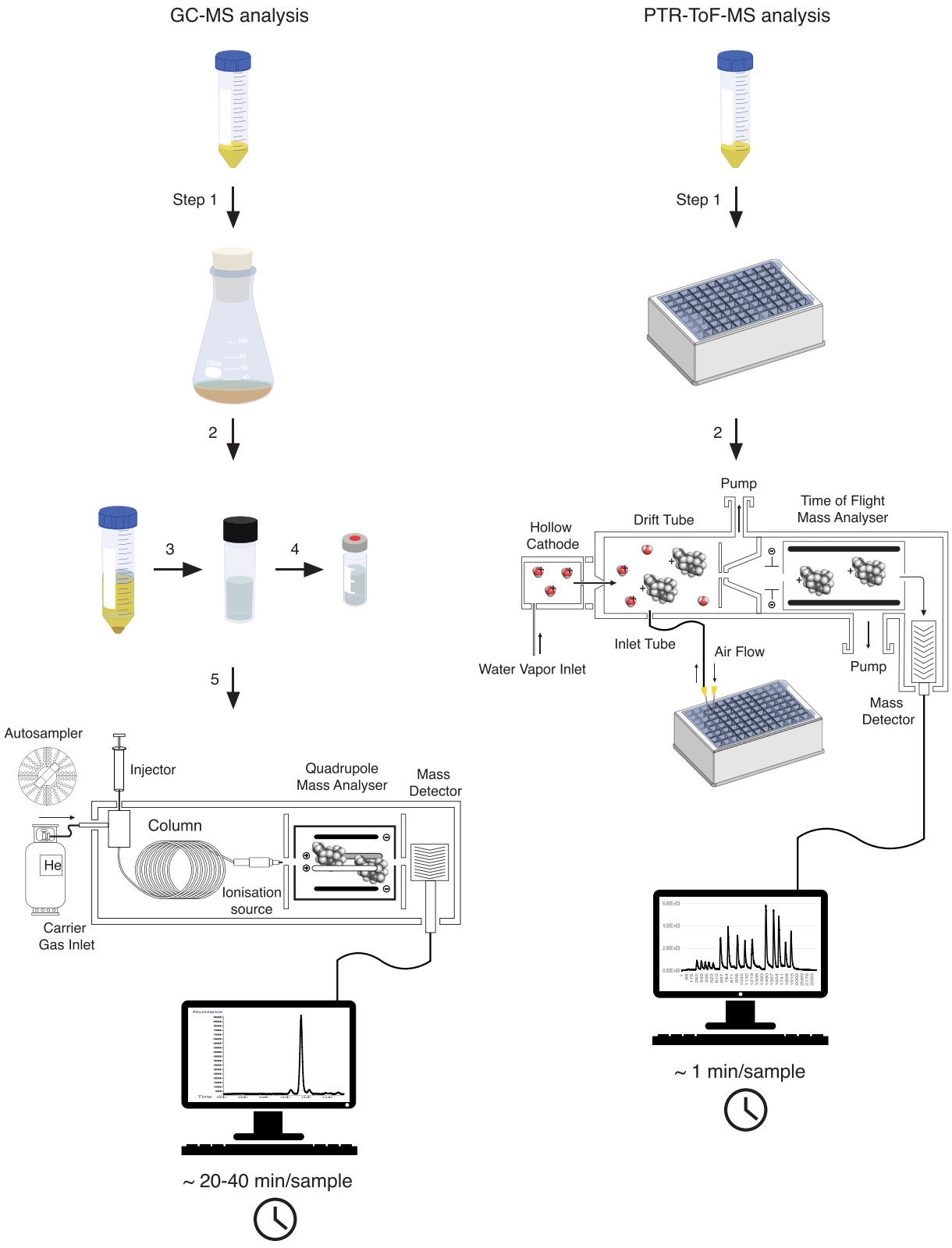

FIGURE 5 Schematic representation of steps and terms involved in GC-MS versus PTR-ToF-MS analysis. The steps necessary to complete the analysis are reported (5 for GC-MS against 2 for PTR-ToF-MS), together with the averaged times of analysis per sample using the two different methods.

production and accumulation has not yet been investigated, and this might be an important factor to consider in the choice of the timing for the measurements. Interestingly, the NCPS values in 250 and 100 mL bottles were in the order of $10^5/10^6$ (Figure 2B), whereas the smaller scale incubations in dw plates result in NCPS values around 10^3 (Figure 4A–B). A large difference in terpene productivity between these production scales has to be expected, because of the difference in headspace volume. Still, we showed that the relative productivity of different mutants, compared with the wild-type CnVS strain, remained comparable.

PTR-Qi-ToF-MS offers notable advantages in analysis time, which could make the technique attractive for screening of larger terpene synthase libraries. When considering the 96 dw plate setup, 10–15 s per measurement appear to be sufficient to determine the abundance of valencene. If calculating an average of 50 s of recovery time between samples, we can estimate that only 1 min is required for each sample. This translates to the possibility of measuring a 96 dw plate in less than 2 h (Figure 5), which, in turn, opens the possibility to measure about 12 plates in 24 h, for a total of 1152 samples per day. Considering that the current throughput for GC-MS allows the measurement of less than 60 samples per day, we propose that the employment of PTR-Qi-ToF-MS as an HTS could bring considerable benefits. For this to be feasible, it is however essential to implement the PTR-Qi-ToF-MS with an ad hoc autosampler, which can allow for a better measurement standardization with a full automation of sampling processing.

Despite the advantages presented by the use of PTR-Qi-ToF-MS, it is important to make some remarks. First, as mentioned above, it is crucial to establish an automated system for the successful application of this technique for HTS of large mutant libraries, as it has been proposed and applied in food research.[34] The system should be able to optimize both the measurement and the recovery time and guarantee consistency in the measuring process. Ideally, an automated liquid handler should also be implemented for the preparation of the dw plates, as described by Leferink et al.[3]

Second, the variation observed between the replicates must be considered when designing the sampling scheme. The design includes five replicates per sample. To make the design efficient for HTS applications, the number of replicates must be lowered. Although an autosampler might help minimize this variation, the design should include at least three replicates per mutant, as tested for the 24 dw plates. Furthermore, to reduce the memory effect and keep the contamination under control, a blank sample should be included between each set of replicates. A 96 dw plate design appears to have good potential for HTS application. In fact, no major reduction in growth was detected in comparison with the other conditions (Figure 4F), and no issues were encountered with the measurements of the different wells in comparison with the 24 dw plates.

Finally, we must consider that, as for most of the HTS methods, PTR-Qi-ToF-MS can only be used to identify preliminary candidates. In fact, although GC-MS analysis allows the identification of different isomers, PTR-Qi-ToF-MS only provides a quantification of the total signal for each mass peak. Although previous studies showed that differentiation between compounds with the same mass is possible,[35,36] it is difficult to reliably quantify each compound in a mixture. Further experiments may provide a method to distinguish between isomers; however, this implementation is beyond the scope of the present work.

Here, we present a method to screen for improved enzyme(s), compared with a well-characterized wild type, which allows a substantial reduction of screening time (Figure 5). Following the complementarity principle introduced by Majchrzak and colleagues,[37] a GC-MS analysis of selected mutant strains will be necessary to obtain the full characterization of the mutants of interest.

5 | CONCLUSIONS

In this study, we propose the application of the PTR-Qi-ToF-MS as an HTS method for the selection of improved (sesqui)terpene synthases. The successful miniaturization to a 96 dw plate setup for bacterial cultures provides a promising starting point for the implementation of this technique for the screening of large libraries of mutants. Our results provide a feasibility assessment, but optimization is still necessary to routinely employ PTR-Qi-ToF-MS for this purpose, as the application for HTS depends heavily on efficient automation. The versatility of the method clearly allows its extension to the detection and screening of other volatile compounds produced in microbial hosts, not only for enzyme engineering but also for strain engineering. Our study provides therefore a proof of principle for a novel HTS method for the reliable selection of the better performing VOC-producing enzymes in a library of mutants.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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