Hungarian University of Agriculture and Life Sciences

# METHOD DEVELOPMENT AND EVALUATION FOR SAMPLING, HEADSPACE GC-MS ANALYSIS AND PREDICTIVE USE OF VOLATILE ORGANIC COMPOUNDS FROM ECONOMICALLY IMPORTANT PLANTS

***Thesis of the Doctoral (PhD) Dissertation***

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## INTRODUCTION - IMPORTANCE OF THE SUBJECT

Within the realm of agricultural research lies a captivating exploration of plant volatile organic compounds (VOCs) and their pivotal role in signaling stressors and physiological changes. Numerous research groups have dealt with the detection of plant infections, separation from healthy plants, and analysis of VOCs (Jansen et al. 2010, Elad et al. 2016, Kasal-Slavik et al. 2017). Pathogen-derived BVOCs (biogenic volatile organic compounds) can substantially and dynamically modify the VOC profile in and above a crop field or even on a larger scale and may also function as biomarkers for the detection of or forecasting early infections (Li et al. 2019). However, surprisingly little is known at present about the composition and quantity of BVOC emission specifically from crop fields (Guenther 2013, Bachy et al. 2016 and 2020), which is in contrast with their comparatively great abundance. Therefore, it is of high priority that their precise composition, temporal and geographical distribution, and fluxes are characterized and understood. Embedded within the E-nose laboratory at the Centre for Agricultural Research in Martonvásár (Hungary), our group aimed at discerning the intricate odor compositions emitted by agricultural plants to facilitate early detection of pests and pathogens through scent alterations. From the existing sampling techniques, non-invasive ones can be either from static or dynamic headspace (DHS). Pull-type open-loop DHS collection followed by solid-phase extraction (SPE) for liquid sample generation and subsequent GC-MS untargeted analysis is an approach readily used by many scientific fields. However, performance testing and evaluation studies (required for most analytical methods) are scarce at best. Adaptation and testing, even further developments are possible for this approach. The methodological approach has been applied for different plant hosts and pathogens to establish biomarker biogenic volatile organic compounds (BBVOCs) corresponding to distinct adverse states across various stages of plant growth and infection. GC-MS data were organized into a robust database housing relevant VOC patterns serving as the cornerstone for subsequent machine learning model construction.

Wheat-powdery mildew (PM) interaction was tested and presented since wheat is the most important cereal in the temperate climate (ca. 750 million tons harvested on more than 200 million hectares globally). PM disease, caused by *Blumeria graminis* f.sp. *tritici* (*Bgt*), is one of the most widespread foliar diseases of wheat globally. It occurs practically everywhere wheat is grown, and thus may release biomarker and other BVOCs from millions of hectares worldwide. Yield reductions without protective measures may amount, in extreme cases, to 40-50% (Savary et al. 2019), while grain quality is also affected (Gao et al. 2018). This is an obligate biotrophic pathogen, *i.e.*, it grows only on the leaves of living plants. Importantly, BVOC emission from wheat (Bachy et al. 2020) appears to be weak and simple in profile compared to other crops (Gomez et al. 2019). This relatively "noise-poor" volatile background provides a yet unnoticed advantage and represents an excellent experimental system to screen for specific BVOCs that are diagnostic biomarkers, therefore BBVOCs and may be involved in and signal the progression of *Bgt* or other fungal pathogen infection in wheat and other cereals (Hamow et al. 2021).

## MATERIALS AND METHODS

**1. Pilot experiments for surveying different plant-pathogen/pest setups and methods for VOC collection,** The term "n" refers to number of replicates (Radványi et al. 2019)

| Plant Species | Cultivar | Growth Conditions | Infectious agent | Sampling time, and approach, duration and temperature |
|---|---|---|---|---|
| Wheat (*Triticum aestivum*) | 'Carsten V' | 18-20°C, long-day | *Blumeria graminis* f. sp. *tritici* type 51 | (7 days after inoculation - DAI) and in advanced disease stage (14 DAI); n=8, DHS sampling (Porapak Q), 24 h, 25-30°C |
| Barley (*Hordeum vulgare*) | 'Harrington' (BC 52), 'Mv Initium' (BC 5), 'KH Hunor' (BC 168) | 25°C, long-day | *Pyreno-phora teres f. teres* | Harrington: 7 DAI (n=1 for control, wounding and inoculated), and 20 DAI (n=2 for control and inoculated); Mv Initium: 8 DAI (n=1), KH Hunor: 23 and 37 DAI (n=2) DHS sampling (Porapak Q), 24 h, 25-30°C |
| Tomato (*Solanum lycopersicum*) | 'Uno Rosso' F1 | 25°C, natural light | *Botrytis cinerea* (B0510) | In the visibly advanced stage of the disease. SPME (50/30 µm DVB/CAR/PDMS), 30 min, 25-30°C |

**2. Plant material and inoculation treatment of wheat – PM VOC surveys in greenhouse and growth chamber experiments**

Seeds (20-30 pieces) of the susceptible bread wheat cultivar 'Carsten V' were sown each into 1-liter clay pots containing garden soil and 1 cm of sand layer on the top. The bread wheat cultivar 'Carsten V' was used in all but one experiment because it does not contain any known *Pm* resistance genes to powdery mildew (Vida et al. 2002) and therefore it is expected to be susceptible to all *Bgt* pathotypes. Plants were grown in an automated greenhouse at a humidity of 60-90%. To simulate environmental temperature variations, three independent experiments were carried out in January-February of 2018 (28 days) and February-March of 2019 (31 days) and 2020 (29 days). Temperature was continuously recorded in 10-min intervals inside as well as outside the greenhouse compartment. DHS

sampling duration was 24 h for all samples (Hamow et al. 2021). Inoculation was applied by manually shaking conidiospores of *Bgt* pathotypes 51 and 71 onto single leaves of 7-days-old test plants (stages 11-12 at the Zadoks scale, Zadoks et al. 1974) in a closed box. During May 2020 two wheat cultivars ('Mv Suba' and 'Mv Kolompos') were grown in a reach-in plant growth chamber according to the T2 spring program (Tischner et al. 1997). Plants were inoculated at the beginning of flowering with a conidiospore mixture of *Fusarium graminearum*/*F. culmorum*, but spontaneously showed slight PM symptoms about 15 days later only on plants weakened by *Fusarium* disease. To test wider applicability and robustness of the identified BBVOCs in further cultivar-pathotype combinations, sampling of eight healthy control and eight *Fusarium*-inoculated and *Bgt*-infected plants for both wheat cultivars was carried out for 8 h (Hamow et al. 2021).

**Experimental setup** and numbers of independent replicate samples from headspaces of *Bgt*-inoculated wheat plants in the greenhouse ('Carsten V') in three consecutive years (2018-2020) and in a growth chamber ('Mv Suba' and 'Mv Kolompos', 2020)

| | | Greenhouse | | | | Growth chamber | **Grand** |
|---|---|---|---|---|---|---|---|
| Treatment | DAI | 2018 | 2019 | 2020 | Subtotal | Treatment total | 2020 | **total** |
| Control | 7 | 4+4 | - | 4+4 | 16 | 40 | 8+8 | **56** |
| | 14 | 4+4 | 4+4 | 4+4 | 24 | | | |
| Inoculated | 7 | 4+4 | - | 4+6* | 18 | 48 | 8+8 | **64** |
| | 14 | 4+4 | 6*+6* | 4+6* | 30 | | | |
| Subtotal | | 16+16 | 10+10 | 16+20 | 88 | 88 | 16+16 | **120** |
| Total (Year) | | 32 | 20 | 36 | | | 32 | |
| Analysis type | | untargeted | untargeted | targeted | | | untargeted | |
| (VOC no.) | | (48) | (48) | (6) | | | | |

DAI: days after inoculation; *four replicate inoculations by pathotype 51 + two replicates by pathotype 71

## 3. Open-loop pull-type-DHS VOC collection and SPE type sample preparation

Solvents used were HPLC/GC grade. The 97 reference materials utilized were obtained from the Merck-Sigma group and (5Z)-octa-1,5-dien-3-ol was purchased from Toronto Research Chemicals. The Porapak Q adsorbent for VOC trapping was supplied by Waters Corp. and 50 mg was inserted into each VOC trap. From the available reference materials, 1 mg $ml^{-1}$ stock solutions were prepared (used for dilutions and mixtures) in borosilicate PTFE-capped screw-top centrifuge tubes, which were stored at -20°C. In each experiment, external solvent-based calibrations were applied. Requirements for calibration and analyses were fulfilled as set by SANTE/11312/2021 (Guidance Document on Analytical Quality Control and Method Validation Procedures for Pesticide Residues Analysis). Unless indicated otherwise, this was applicable for 0.1-2.5 μg $ml^{-1}$ (ng $ml^{-1}$corresponded to 0.1-2.5 ng on column injection) centration range using at least three points for calibration. For recovery experiments, 1 μg $ml^{-1}$ spiking was applied (for elution volume). After spiking/sampling/etc. events sorbent tubes were eluted with 300 μl of *n*-hexane, traps were cleaned before and after use with solvents, and were purged with $N_2$ flow. For internal standard 1-bromodecane was used, spiked to the eluate right after elution of the sorbent into a vial. For spiking 100 ng was added in 2 μl of *n*-hexane. Continuous DHS sampling was done in all experiments for either 6 or 8 h, during greenhouse experiments for 24 h with 0.8 L $min^{-1}$ flow rate. The establishment of a closed headspace was done from borosilicate special glassware or PTFE bags around the plants/plant parts sampled. In the case of periodic DHS sampling events (for desorption loss characterization), the flow was switched „on" every 5 min, followed by „off" for 10 min sequentially using „nose-e", a locally developed prototype portable VOC collection apparatus. The sampling duration was 6 h at 26 °C. Sampling temperatures in other experiments were not controlled but registered during sampling and plant cultivation. For binding site competition tests

homogenized fruit of commercially available pear and/or tomato was used with either or both present inside the headspaces sampled.

**4. GC-MS VOC analysis method for liquid injection of eluates and samples obtained by open-loop-pull-type-DHS VOC collection and SPE elution sample preparation**

GC-MS analyses of liquid samples were carried out on an Agilent 7890B GC coupled to a 5977B MS system. The instrument was equipped with a Gerstel MPS CTC-type autosampler and a CIS4 inlet with a septumless head installed. Injection volume was 1 µL in splitless mode (for SPME samples split mode, split ratio 1:10), septum purge flow was 3 mL $min^{-1}$, and purge (for SPME split) flow was 50 mL $min^{-1}$ starting from 3 min. Before each run the CIS4 inlet was cooled with liquid $CO_2$ to 20 °C and the temperature was equilibrated for 0.5 min. The injector temperature program was set as follows: 20 °C, held for 0.25 min (initial time), then increased with a rate of 12 °C $sec^{-1}$ up to 270 °C with a hold time of 6 min. Separation was carried out on a J&W HP-5MS UI 30 m x 0.25 mm x 0.25 µm semi-standard, non-polar type capillary column (Agilent). Helium 6.0 was used as a carrier gas with a flow rate of 1 mL $min^{-1}$ (36.26 $min^{-1}$) in constant flow control mode. The oven temperature program was set as the following: 40 °C hold for 3.5 min, increased by 7 °C $min^{-1}$ to 140 °C, then by 20 °C $min^{-1}$ to 280 °C and held for 2 min. For MS detection EI ionization was used with a standard 70 eV energy. The auxiliary heater was set to 250 °C, MS source to 250 °C and MS quad to 150 °C. Mass spectra were collected in scan and SIM combined acquisition setting, and solvent cut time was 5.2 min.

**5. Data evaluation and mining, statistical analysis**

Mass Hunter Workstation Qualitative Navigator B.08.00 and Quantitative Analysis B.09.00 software tools (Agilent) were used for evaluation and quantitation. Compound identification was based on background-subtracted mass spectra by the NIST MS Search program and MS spectral and retention index (RI) Library v17, the Wiley Registry® of MS Data, 10th edition, and by utilizing *n*-alkane RI with a C7-30 *n*-alkanes mix. The highest-ranked consistent library hit (min. 75% similarity with the reverse search for mass

spectra) and RI score match were accepted for the identification of volatile compounds. Integration was carried out for the most abundant unique ion for each peak. For peak areas lower than the limit of quantification (LoQ), the background was always recorded with non-zero values for reliable statistical tests. The statistical significance of the differences between controls and treatments (symptomatic stages, pathotypes, and years) was analyzed by two-sample *t*-tests as well as by multivariate PERMANOVA. To identify potential VOC biomarkers, principal component analysis (PCA) was applied for unsupervised reduction of data dimensions after standardization by *z*-score normalization of the original data matrix (Hamow et al. 2021).

## RESULTS AND DISCUSSION

### 1. RESULTS OF THE PILOT EXPERIMENTS FOR SURVEYING DIFFERENT PLANT-PATHOGEN SETUPS AND METHODS FOR VOC COLLECTION

The first aim primarily sought testing and selection of a non-invasive static/dynamic sampling and analysis approach founded on trial experiments. Investigation into various barley cultivars revealed distinct aroma profiles. Mechanical damage induced the appearance of green leaf volatiles (GLV) such as (*Z*)-3-hexenyl acetate.

In the case of *P. teres*-infected barley, new compounds appeared on the chromatograms compared to controls. Varied trends in compound intensity were observed, signifying potential markers for infection stages. Similarly, the analysis of tomato odor profiles concerning gray rot and especially wheat powdery mildew interaction that our group focused on identified promising BBVOC candidates (Radványi et al. 2019).

## 2. RESULTS OF PULL-TYPE OPEN-LOOP DHS-SPE-GC-MS METHOD PERFORMANCE TESTS AND THEIR INTERPRETATION

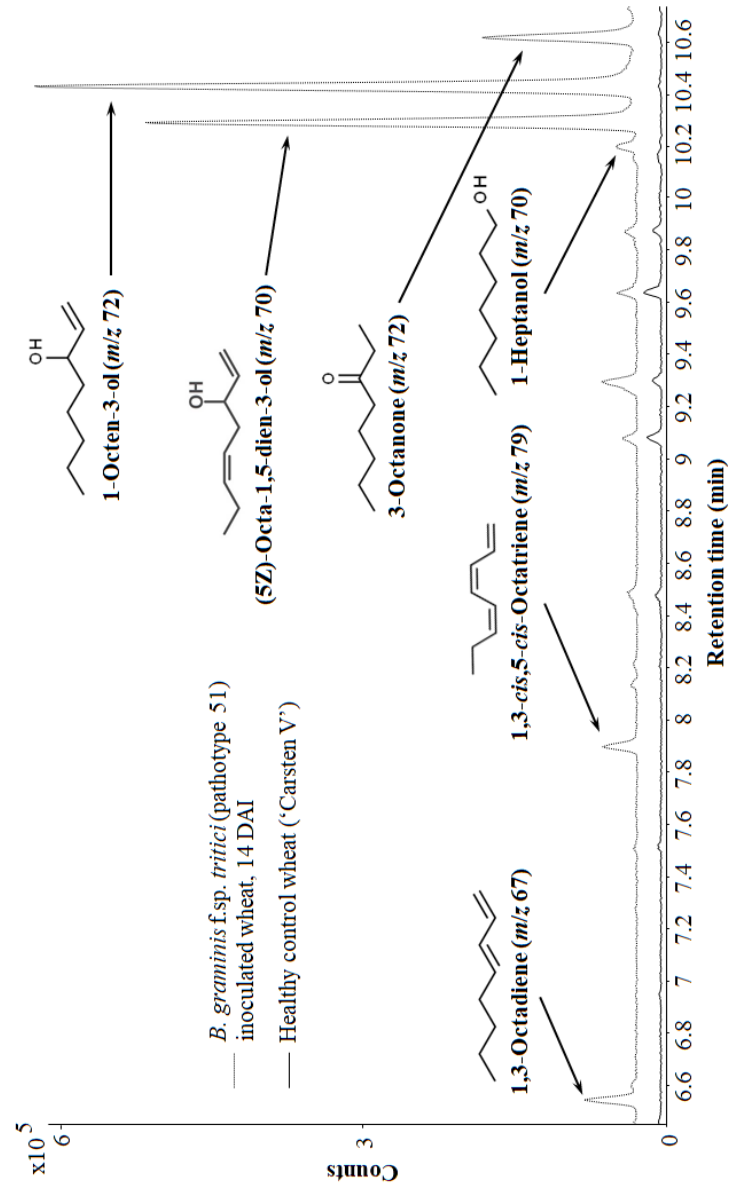
2.1 The methodological approach that was adopted and tested for compliance with the qualitative and quantitative analytical requirements was conform with the SANTE/11312/2021 guidelines that are used for pesticide residue analysis. Adsorbent SPE elution recovery with and without 1-bromodecane internal standard correction has been tested. Findings about the direct spiking of VOC traps by reference mixes, SPE elution, and recovery assessment suggested that recovery may have been overestimated. Recovery meant that if internal standard correction was not applied to the elution volume, the quantitative accuracy of concentration was overstated by 40–60%. 87 VOCs' average recovery in the internal standard correction scenario involving 96 chemicals was categorized as being over 60%. Recovery ranged from 40% to 60% for 2-methyltetrahydrofuran-3-one, methyl benzoate, 1,3-dimethoxybenzene, α-terpineol, (S)-(+)-carvone, eugenol, and methyl eugenol. Recovery for methyl jasmonate and ethyl 3-hydroxybutyrate was between 20% and 40%.

2.2 Regarding the second aim set, additional tests included characterizing the effects of sorbent breakthrough and desorption through recovery experiments by comparing continuous and novel periodic DHS-VOC sampling. This could be a potential improvement to the approach that aimed to reduce total flow volume by periodically starting and stopping the flow, thereby mitigating potential breakthrough and desorption losses. These were observed primarily in the case of low-boiling-point compounds that are early elutes in the C8-C9 elution region and compounds containing hydroxyl groups, such as alcohols. Periodic DHS displayed a trend of slightly higher recoveries with less deviation, but further optimization (sampling duration, cycle times, flow rates, adsorbent quality and quantity, temperature of VOC traps, and elution solvent type for SPE) would be advisable in the future.

2.3 As a final test, it was determined that even at high background levels, the VOC traps applied have adequate capacity for trapping and even additively semi-quantify different strong smells coming from various sources in the closed headspaces sampled. This is in line with the second aim-set evaluation of sorbent trap capacity and the competition of VOCs for the volatile trap binding sites for continuous DHS sampling.

## 3. RESULTS OF THE EMISSION OF NOVEL VOLATILE BIOMARKERS OF WHEAT POWDERY MILDEW

The third aim involved the application of this methodology to explore the interaction and discovery of BVOCs in wheat powdery mildew (*Blumeria graminis* f.sp. *tritici*, *Bgt*). Six novel BVOCs as indicators of infection in early and advanced states have been identified. Three minor (1,3-octadiene, 1,3(Z),5(Z)-octatriene, 1-heptanol), and three major BBVOCs (1-octen-3-ol, 5Z-octa-1,5-diene-3-ol, and 3-octanone) were identified and proposed as biomarkers. Biomarker BVOC emissions (in various abiotic conditions, genotypes, mixed pathogen backgrounds, and different plant growth stages and years) increased with disease severity from early (7 DAI) to advanced stages (14 DAI). To sum up, the pull-type DHS-SPE-GC-MS methodology has proven to be a flexible way of differentiating healthy wheat plants from those that have powdery mildew (Hamow et al. 2021). The creation of VOC databases for machine learning-based prediction model development made use of collected datasets. With a 99.7% accuracy, a Random Forest-based model produced the best results for VOC-based distinction between healthy plants and those affected by powdery mildew, the six proposed BBVOCs classified as the highest ranked components responsible for differentiation. This provides additional support for pathotyping and prediction; using VOC fingerprinting, early disease identification can be accomplished (Hamow et al. 2021).




Pooled extracted ion chromatograms of the optimal unique mass peaks to compare the six marker VOCs between samples collected from healthy (base line) and *Bgt*-inoculated (upper line) wheat plants.

## CONCLUSIONS AND SUGGESTIONS

Static and dynamic VOC collection methods followed by untargeted GC-MS analyses resulted in the differentiation of healthy and diseased plants based on VOC fingerprints. The pull-type open-loop DHS SPE sample collection and preparation method adapted, and performance tested thoroughly has limitations and challenges. Accuracy of quantitative comparison and quantitation for nearly 100 VOC compounds were affected by compound SPE recovery. Compounds can exhibit varying amounts of losses, effect of calibration mixtures would be advisable to be better characterized in future research. Use of internal standards for elution volume correction enhances quantitative accuracy by 40-60%. Combining static and dynamic methods and analysis approaches as an integrated in-situ system, as proposed by Duc et al. (2022), could facilitate a more in-depth characterization of VOC fluxes and their origins in the future. Optimization of sampling setups and promising novel approaches like periodic DHS sampling would be recommended (sampling duration, cycle times, flow rates, adsorbent quality and quantity, temperature of VOC traps and elution solvent type for SPE). Through the interaction between wheat powdery mildew and VOCs, unique biomarker BVOCs were found, which may help with patho- and chemo-diagnosis as well as resistance breeding. Hundreds of tons of these BBVOCs with variable half lifetimes may have been released into the atmosphere based just on the projected BVOC emission from the wheat-powdery mildew interaction. The identified BBVOCs may have aided in the creation of secondary organic aerosols by taking part in atmospheric processes such as the catalytic breakdown of tropospheric ozone. Therefore, the scientific community should give it a top priority to reevaluate and carry out research to determine the precise composition, geographic distribution, impact, and fluxes associated with BVOC emissions from the agri-environment for the most significant diseases and fungal pathogens of cultivars grown in large areas of the world.

## NEW SCIENTIFIC RESULTS

1. Tested and critically evaluated (based on SANTE/11312/2021 guideline) the performance of the Porapak Q adsorbent was for nearly a hundred different reference compounds using an open-loop-pull-type dynamic headspace VOC collection, n-hexane SPE elution, and GC-MS analysis method. This work has never been done to such an extent before.
2. Proved the value of applying 1-bromodecane as an internal standard when *n*-hexane is used to elute an adsorbent's SPE. Concentrations between 40% and 60% are the consequence of overestimating recoveries in the absence of internal standard correction. However, 90% of the 96 compounds examined showed an average recovery of 60% or above after accounting for the elution volume using 1-bromodecane.
3. Introduced the concept of periodic DHS technique (self-developed refinement). Recovery trials for both continuous and periodic DHS-VOC monitoring for 96 compounds exhibited losses for alcohols and low-boiling-point VOCs that might be minimized by periodic DHS. Desorption effects and breakthroughs have been attributed to these losses.
4. Demonstrated by testing the adsorbent trap capacity and competition of components for adsorbent binding sites during continuous DHS sampling that even in cases of high background, no competition was observed, and the quantitative abundance of VOCs trapped from different emission sources in the sampled headspace (tomato and pear fruit) could be considered additive.
5. Successful BVOC-based differentiation of healthy and *Bgt.* powdery mildew infected wheat plants, by characterizing biomarker biogenic volatile organic compounds (BBVOC) as indicators of infection. Minor BBVOCs discovered were 1,3-octadiene, 1,3(Z),5(Z)-octatriene and 1-heptanol. Major novel BBVOCs were (5Z)-octa-1,5-diene-3-ol, 1-octen-3-ol and 3-octanone. The emission of these BBVOCs increased with disease progression and severity.

6. Proved that BBVOCs of *Bgt* are robust in various environmental conditions, years, and even in mixed pathogen background (*Fusarium* inoculated wheat genotypes also infected by PM).
7. Revealed that biomarker and possibly other BVOCs related to *Bgt.* were estimated to be emitted by agroecosystems in massive quantities (ca. 188 metric tons per month), possibly participating in affecting atmospheric processes significantly by forming secondary organic aerosols.

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