Title: Screening of leaf extraction and storage conditions for eco-metabolomics studies

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Highlight

We developed an on-site metabolite extraction method for leaf tissue samples from field studies in challenging logistical circumstances. We highlight extract stability and reproducibility compared to frozen or dried tissue.

Abstract

Mass spectrometry-based plant metabolomics is frequently used to identify novel natural products or study the effect of specific treatments on a plant's metabolism. Reliable sample handling is required to avoid artifacts, which is why most protocols mandate shock freezing of plant tissue in liquid nitrogen and an uninterrupted cooling chain. However, the logistical challenges of this approach make it infeasible for many ecological studies. Especially for research in the tropics, permanent cooling poses a challenge, which is why many of those studies use dried leaf tissue instead. We screened a total of ten extraction and storage approaches for plant metabolites extracted from maize leaf tissue across two cropping seasons to develop a methodology for agroecological studies in logistically challenging tropical locations. All methods were evaluated based on changes in the metabolite profile across a 2-month storage period at different temperatures with the goal of reproducing the metabolite profile of the living plant as closely as possible. We show that our newly developed on-site liquid-liquid extraction protocol provides a good compromise between sample replicability, extraction efficiency, material logistics, and metabolite profile stability. We further discuss alternative methods which showed promising results and feasibility of on-site sample handling for field studies.

Keywords

UHPLC-MS, chemical ecology, agroecology, Maize (*zea mays*), extract stability

1. Introduction

In agriculture, high-throughput phenotyping approaches have become essential to assess traits related to increased yield, as well as those that confer tolerance to environmental stresses in crops (Araus and Cairns, 2014). Metabolomics is a powerful analytical approach that can provide information on the patterns and nature of plant responses to the environment, by providing information on the chemical features, identity, and quantity of metabolites produced by plants in different conditions (Sardans *et al.*, 2021). In this way, metabolomics can add the chemical dimension to the high-throughput crop phenotyping toolbox, as thousands of metabolic markers often representing hundreds of metabolites can be recovered from a single leaf sample (Brunetti *et al.*, 2013). Investigations of plant stress responses commonly focus on specialized metabolites, which are not essential for cell growth and development and are instead synthesized or modified by plants in response to specific environmental triggers (Macel *et al.*, 2010; Walker *et al.*, 2022).

Nevertheless, high-throughput phenotyping platforms have been developed under refined conditions (i.e., greenhouse and growth chamber facilities proximate to laboratories) and only reliably work with specialized equipment, which limits their application when dealing with realistic (field) conditions (Araus and Cairns, 2014). Such limitations extend to the use of a metabolomics approach in agriculture, where sample preparation and storage is a crucial step towards obtaining high quality data. For instance, most protocols in plant metabolomics require liquid nitrogen to shock-freeze the tissue immediately upon collection and keep the material frozen during the sample handling procedure. While this approach offers the closest representation of the metabolites in the living plant, it requires uninterrupted cooling (usually at -80 °C) and rapid sample handling to avoid thawing and degradation (Ossipov *et al.*, 2008; Sedio *et al.*, 2018; Bakhtiari *et al.*, 2021).

A common alternative, when cooling conditions are not meet, is to dry the plant tissue on-site and then transport and store the dried material (Fernandez-Conradi *et al.*, 2022). This allows for reproducible sampling with minimal material requirement; however, little data is

available on how the drying process changes the obtained metabolite profile due to differential stability of different metabolites. As a result, there is a need for a sample preparation method that ensures sample stability until the samples can be processed in the laboratory. This is particularly relevant when the sampling fields are located far from the laboratory facilities, and field campaigns are not easy or possible to repeat.

Here, we address limitations for the use of metabolomics in realistic agroecological conditions by describing and comparing sample handling methods. These methods were conceived in the context of a larger project aiming at understanding the metabolomic profile of maize grown under different conditions in tropical Africa, where weather and logistics conditions can make a metabolomics approach challenging. We first evaluated the suitability of two leaf preservation and six extraction methods, based on changes in metabolite profile across a 75-day storage period, to determine the method that resulted in the best apparent sample stability as judged by similarity to the metabolite profile obtained by the laboratory standard procedure: solid-phase extraction, or liquid-liquid extraction of flash-frozen and finely powdered leaf tissue within a day after harvest. We then conducted a follow-up study focussing on an on-site liquid-liquid extraction procedure in comparison to in-field air-drying followed by laboratory extraction, and the laboratory standard procedure. Our results demonstrate that this liquid-liquid extraction procedure generates reproducible metabolomic profiles while being feasible for field studies in terms of effort and stability of extracts. The methodology presented in this paper has the potential to be a viable alternative to the more established methods for plant metabolomics research in field studies and contribute to a better understanding of plant metabolism under realistic conditions.

2. Materials and Methods

2.1. Chemicals and materials

Acetonitrile (MeCN), methanol (MeOH) and isopropanol were obtained from *Biosolve* (ULC grade, Valkenswaard, Netherlands) and formic acid from *VWR Chemicals* (LC–MS grade, Dietikon, Switzerland). Ultrapure water (< 2 ppb TOC) was produced using a Milli-Q Advantage

A10 water purification system (*Merck*, Burlington, MA, USA). For mass calibration, a 10 mM sodium formate solution was used, and ion mobility calibration was performed using ESI-L low concentration tune mix bought from *Agilent* (Santa Clara, CA, USA). The 10 mM sodium formate solution contained 1 M NaOH (250 µL) and formic acid (50 µL) in 50% isopropanol (25 mL). Dichloromethane (DCM) was purchased from *Honeywell* (Charlotte, NC, USA), Tween-20 from *Fisher Scientific* (Hampton, NH, USA) and all other chemicals from *Sigma-Aldrich* (St. Louis, MO, USA).

2.2. Sample handling for broad method screening

Although we aim to develop a method practical for field research in tropical maize agroecosystems (i.e., central Africa), we required an experimental setting which allowed for comparison to extracts generated with an unbroken cooling chain. For this reason, maize plant tissue was collected from field-grown maize at the Strickhof Competence Centre of Agricultural Sciences (Eschikon, Switzerland, 47.4524090, 8.6806795) and used in eight different sample extraction and storage approaches. An overview of the employed methods is shown in **Figure 1A** and a detailed description of all procedures can be found in SI1.

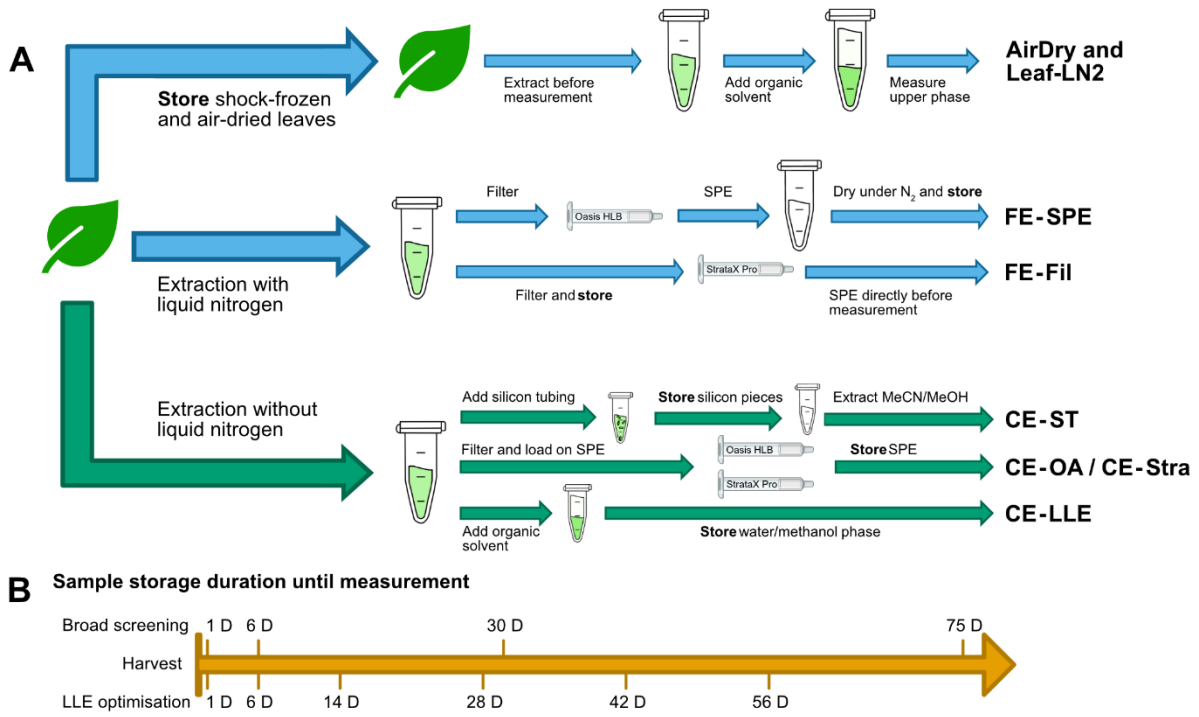

Figure 1: *Overview of the evaluated sample extraction and storage methods. The broad method screening included all methods and samples were evaluated after storage for 1, 6, 30, and 75 days respectively.*

The samples were then stored at three different temperatures (30 °C, 4 °C, and -20 °C) for 1 day, 1 week, 1 month, and 75 days, respectively. At each of those timepoints four replicates of each method and temperature were analysed.

2.3. Sample handling for liquid-liquid extraction optimisation

As a follow up study during the following cropping season, we evaluated metabolite stability in two extraction solutions and compared those results to air-dried and shock-frozen leaf storage. A detailed description of all procedures can be found in SI1. The samples were again stored at the same three different temperatures (30 °C, 4 °C, and -20 °C) and four replicates per timepoint, method and temperature were measured at six timepoints after 1 day to 8 weeks of storage time as shown in the timeline in **Figure 1B**.

2.4. UHPLC-HR-MS/MS Setup

Liquid chromatography was performed on a Vanquish Horizon UHPLC System by *Thermo Fisher* (Waltham, MA, USA) build from a Vanquish binary pump H, a Vanquish split sampler HT and a temperature-controllable Vanquish column compartment. Chromatographic separation was achieved on an ACQUITY Premier CSH C18 Column (130 Å, 1.7 µm, 2.1 × 50 mm, *Waters*, Milford, MA, USA) at 30 °C to reduce column backpressure. Eluent A consisted of H_2O + 0.1% HCOOH and B of MeCN + 0.1% HCOOH. The solvent flow was kept at 0.6 mL/min with the following gradient: (i) 5% B isocratic from 0.0 to 0.4 min; (ii) linear increase to 35% B until 2.8 min; (iii) linear increase to 75% until 3.2 min; (iv) linear increase to 100% B until 3.3 min, (v) holding 100% B until 4.4 min (vi) back to the starting conditions of 5% B until 4.5 min; (vii) equilibration for 1.1 min until the next run. The injection volume is dependent on the employed extraction method and is specified in the detailed extraction protocols in SI1.

A timsTOF Pro hybrid quadrupole-time-of-flight (QTOF) mass spectrometer equipped with trapped ion mobility spectrometry (TIMS) produced by *Bruker* (Bremen, Germany) was connected to the Vanquish UHPLC system and was used to acquire ion mobility and MS/MS data. Ionisation was performed in positive and negative ESI mode and the scan range was set to 20 to 1350 *m/z* at a 12 Hz acquisition rate. Mass and CSS calibration was performed using

the *Agilent* low concentration tune mix (13 compounds in acetonitrile, part number G1969-85020) prior to analysis. For additional mass accuracy, a calibration segment was programmed from 0.05 to 0.15 min at every UHPLC run with the help of a 6-port-valve with a 20 µL loop which contained a solution of 10 mM sodium formate clusters.

2.5. Software and Data Treatment

Instrument control was done using Hystar (*Bruker*, version 6.0) containing a Chromeleon Plug-In (*Thermo Fisher*, plugin version 1.3.8, Chromeleon version 7.3.0) and otofControl (*Bruker*, version 6.2). Data quality assessment was performed in DataAnalysis (*Bruker*, version 5.3) and data treatment (detailed below) in MetaboScape (*Bruker*, version 2022b). Figure plotting was done using python (version 3.8.5) in the Spyder IDE (version 5.0.3) using the libraries pandas (version 1.2.4), and bokeh (version 2.3.2). Posthoc analyses were performed with R (version 4.2.2) with the library emmeans (version 1.8.3).

MetaboScape was used for peak picking, blank subtraction, data normalisation by internal standard, Pareto transformation, and data evaluation with principal component analysis (PCA). The effects of Pareto transformation were checked on representative datasets to ensure that this normalization and transformation resulted in a similar magnitude and approximately normal distribution of metabolite features across samples (Metaboanalyst (Pang *et al.*, 2021), **Figure S1 and S2**). All parameters for the peak picking and data evaluation are shown in SI2. The peak tables were exported in .csv format (see Data Availability) and PCA data was exported in .csv format to plot graphs using our python workflow (see SI3).

2.6. Recommended sample extraction procedure

For the full methods detailing all tested extraction procedures, see the detailed extraction protocols in SI1. Here, we detail the recommended extraction procedure.

An extraction solution consisting of MeOH / water in a 2:1 ratio and camphorsulphonic acid as an internal standard (20 ng / mL) was prepared, of which 200 µL were added to a 1.5 mL Eppendorf tube for each sample. This solution is appropriate for extracting mid to high polarity metabolites which are commonly studied and contain many specialised secondary metabolites.

Twelve leaf disks were collected with a 6 mm diameter hole punch (*Milian*, Vernier, Switzerland) directly into the extraction solution and the immersion in MeOH directly upon collection may reduce enzymatic activity in the sample (Maier *et al.*, 2010) . The tubes were thoroughly shaken and transported in a common household cooling box containing ice packs.

The leaf tissue was ground inside the Eppendorf tubes using plastic micropestles having a tip with approximately the same volume as the point of the 1.5 mL Eppendorf tubes and attached to a household electric drill. It is recommended to use micropestles with a rough surface to facilitate leaf grinding, which we did by roughening the surface using 240 grit sandpaper. After the leaf tissue was ground to a paste, another 500 µL of the extraction solution was added before shaking thoroughly. The liquid-liquid extraction was performed through addition of 500 µL of chloroform to separate pigments and lipids, followed by thoroughly shaking. After letting the tubes rest for approximately 10 minutes at room temperature, the phase separation was completed, and the upper MeOH / water phase was transferred to fresh microcentrifuge tubes.

3. Results

3.1. Suitability of internal standards

For the broad screening, we selected stevioside as an internal standard, but identified characteristics of the mass spectra during data evaluation which led us to seek alternatives. In the mass spectrum of stevioside in **Figure 2A**, the detected signals for the proton and ammonium adducts (805 and 822 *m/z*) are highlighted alongside the main signal at 319 *m/z* which matches the loss of all three hexose substructures. Additionally, signals were marked which match the loss of one and two hexose substructures.

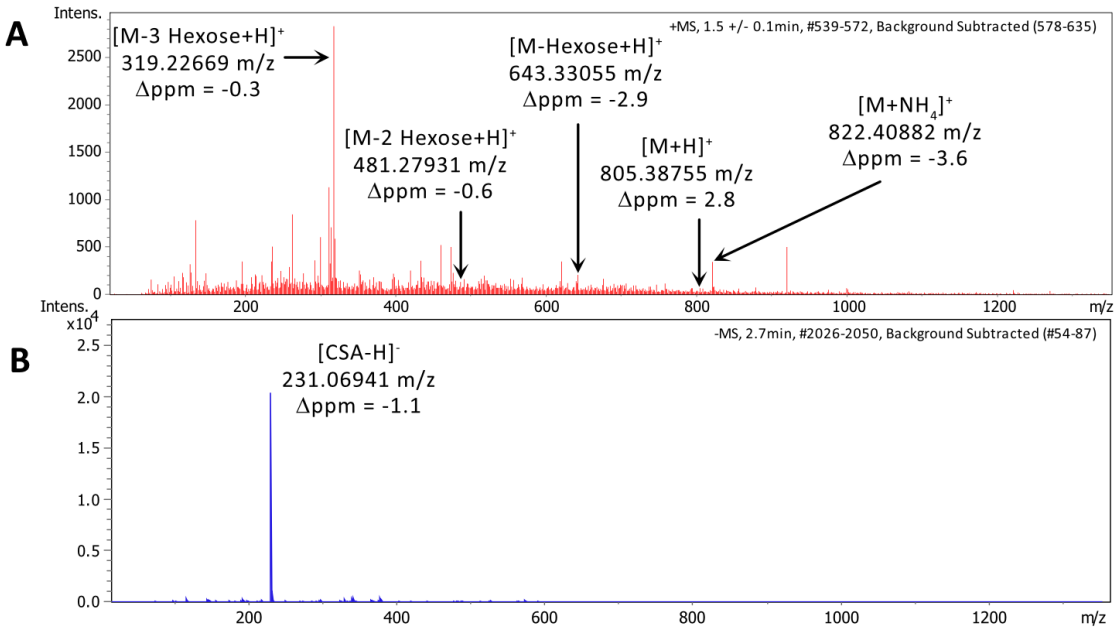

Figure 2: *Comparison of the full scan MS spectrum of the internal standards stevioside (red, A) and camphorsulphonic acid (CSA, blue, B) with signal annotation of matching m/z ratios.*

We attributed this to a possible in-source fragmentation and combined with a slight reduction in peak area observed with longer storage periods, the decision was made to include two additional possible internal standards – camphorsulphonic (CSA) and glycyrrhizic acid – in the narrow method screening experiment. For comparison, the mass spectrum of CSA can be found below the stevioside spectrum in **Figure 2B**, and shows a single signal without any fragmentation. **Figure S3** shows the intensity of each of the three compounds across the storage experiment. CSA showed a stable signal across the storage period and additionally matched better with our target compound mass range, so we recommend using CSA over stevioside or glycyrrhizic acid. For targeted metabolomic analyses, isotopically labelled reference compounds would be preferable.

3.2. Comparison of leaf homogenisation efficiency

Both during the broad method screening and LLE optimisation experiments, different approaches were tested for leaf tissue homogenisation using steel ball mills, ceramic mortars and micropestles. When freezing tissue in liquid nitrogen while grinding, a powder is generally obtained. However, when homogenizing air-dried leaf tissue with either ball mills or ceramic mortars, we were unable to obtain a powder, as some leaf veins remained intact. A direct

comparison of the powders obtained when grinding fresh leaf tissue and air-dried leaf tissue in liquid nitrogen is shown in **Figure 3**.

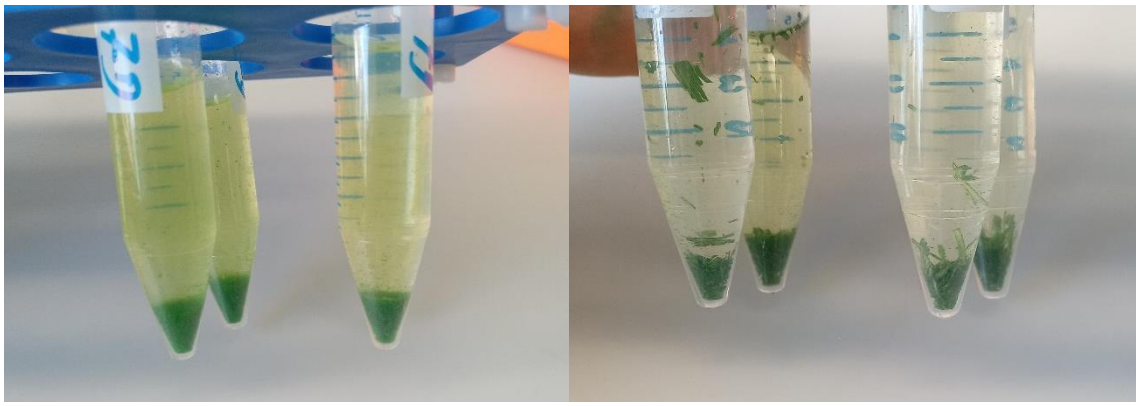

Figure 3: *Comparison of ground flash-frozen (left) versus air-dried (right) leaf tissue following the same pulverization procedures.*

Both of those methods still led to a more homogeneous product than attempting to grind tissue without using liquid nitrogen. Doing so with a ceramic mortar left the leaf tissue structure mostly intact, whereas with a micropestle, a chunky and more homogeneous paste could be obtained (**Figure S4**).

3.3. Selectivity of sample preparation methods

During the broad method screening, fundamentally different sample purification approaches were tested, most notably solid phase extraction (SPE) and liquid-liquid extraction (LLE). The two approaches lead to significant differences in the resulting metabolite profile. In our experiments, the profile after sample workup with SPE was shifted towards molecules with a higher molar mass and a lower polarity compared to samples prepared by LLE. This trend can already be observed in a base peak chromatogram, as shown in **Figure 4**, and becomes clearer when comparing the data in the peak tables. Some signals could only be observed in LLE samples and others only in SPE samples.

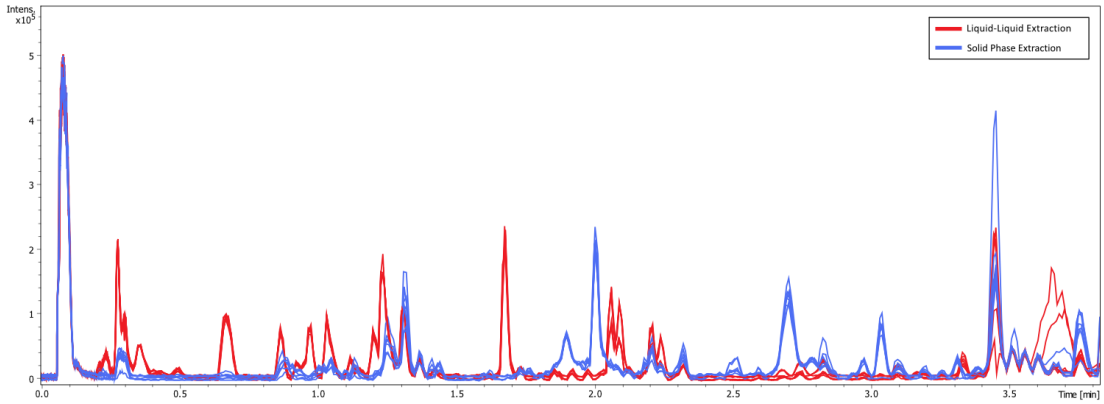

Figure 4: *Overlaid chromatograms of a subset of four samples prepared by solid-phase extraction (SPE, blue) and four samples prepared by liquid-liquid extraction (LLE, red) highlighting generally higher abundance of high-polarity (shorter retention time) compounds when LLE is used.*

The significant shift of the metabolite profile causes a challenge when it comes to multivariate data comparison, where principal component analysis (PCA) is a common approach. Any PCA which contains LLE and SPE samples will group the extraction approaches tightly together as shown in **Figure S5**, which masks the shifts in the profile across a storage period. Thus, all PCA results were plotted separately for LLE and SPE sample groups to allow a sensible interpretation.

3.4. Extract stability over time

Changes in the overall metabolite profile were assessed by PCA, which showed that in almost all cases, a higher temperature led to a greater shift of the profile. During the broad screening, the metabolite profile continued to shift for all evaluated sampling methods without reaching a stable result (which could occur after completing all possible molecular transformations). Examples of the PCA can be found in SI4 with special attention towards **Figures S6** and **S7**, which show the comparison of all evaluated LLE and SPE methods. For the narrow screening the shift of the metabolite profile over time was significantly reduced. As an example, the PCA of all samples prepared using the on-site sample extraction procedure is shown in **Figure 5A**. Notably, samples stored at room temperature are shifted along PC1, while cooled samples (both 4 °C and -20 °C) cluster tightly together. When excluding the room temperature samples, all datapoints cluster randomly in PC1 and 2, while higher PC dimensions show a minor shift over time, which is highlighted in **Figure 5C**. For comparison,

results from storing shock-frozen leaf tissue at -20 °C are shown in **Figure 5D** and demonstrate a shift of the metabolite profile along PC2.

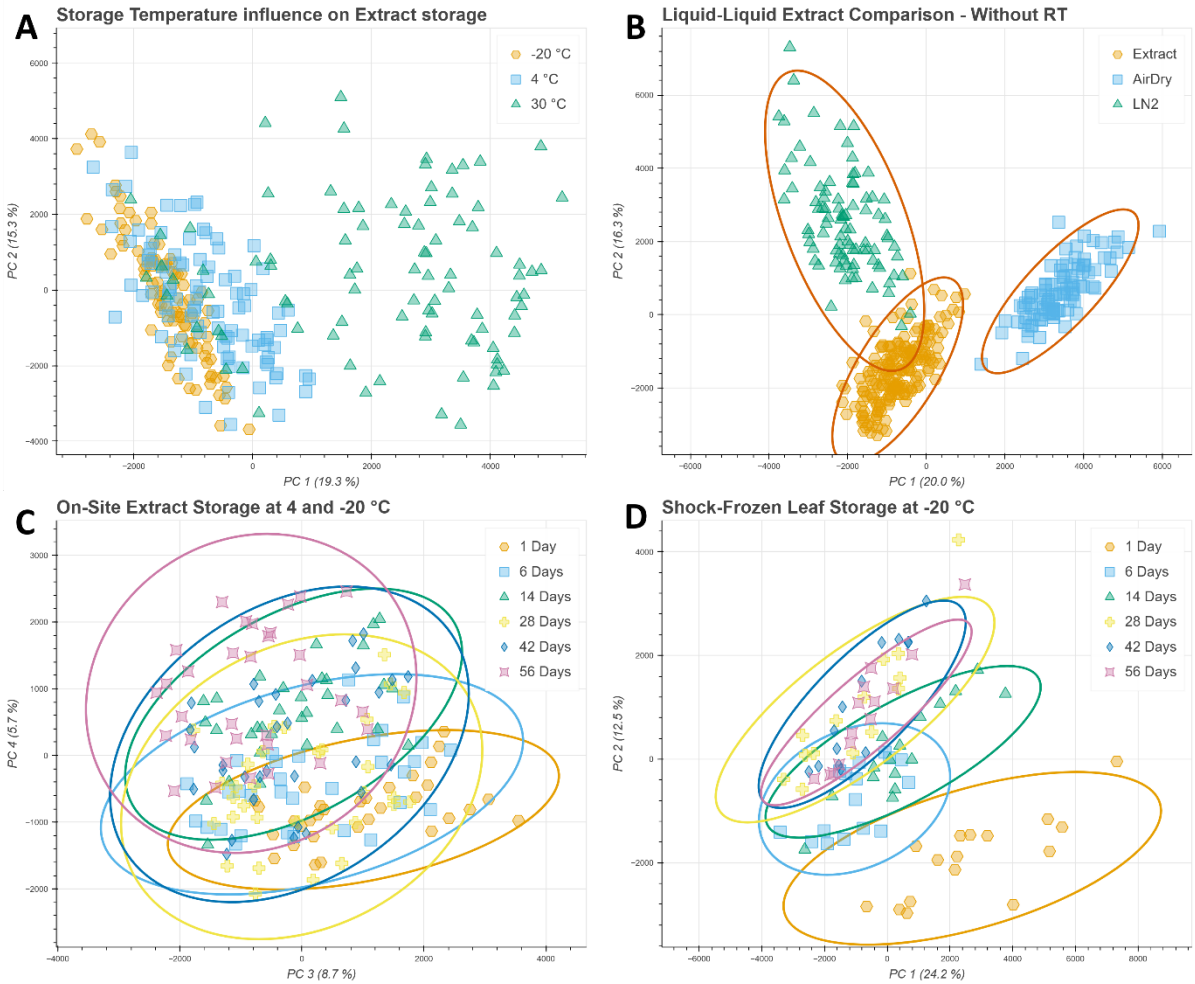

Figure 5: *Principal components analysis (PCA) conducted on metabolite profiles of samples extracted and stored under different conditions: (A) LLE and extract storage at different temperatures, (B) LLE of differently handled leaf tissue samples, (C) on-site LLE and cold storage, (D) frozen storage of shock-frozen leaf tissue.*

The three sample groups screened during the LLE can also be directly compared to each other, which is shown in **Figure 5B**. The samples from air-dried leaf tissue separate along PC1, while the on-site extraction separated from shock-frozen leaf tissue samples along PC2. Notably, the 95% confidence ellipses of the shock-frozen leaf tissue samples and the on-site procedure overlap, while air-dried samples are fully removed from the other two groups. All three groups are significantly different from each other when performing a pairwise comparison based on the first five PCs, but when comparing the on-site extract storage samples to shock-frozen leaf storage, the p-value was found to be 0.0006. Comparing shock-frozen and air-dried

leaf storage returned a p-value below 0.0001, and the same p-value was obtained when comparing air-dried leaf storage to our on-site extract storage (see: SI5, posthoc analysis).

3.5. A note on storage of extracts on SPE cartridges

During the broad screening we found indications that metabolite storage on solid-phase extraction (SPE) cartridges (procedure CE-OA in SI1) could be a viable alternative for on-site sample preparation and storage. **Figure 6** shows the samples stored on the SPE cartridge in comparison to elution and drying the eluent under nitrogen flow prior to storage (procedure FE-SPE in SI1). The samples stored on the cartridge seemed more reproducible (tighter grouping of replicates) and less affected by storage time (less distance between the timepoint groups) than the samples following the “FE-SPE” procedure. Due to material shortages at the time, the “CE-OA” approach was only evaluated at three storage timepoints, and we would therefore recommend more in-depth testing before employing this approach on a larger scale.

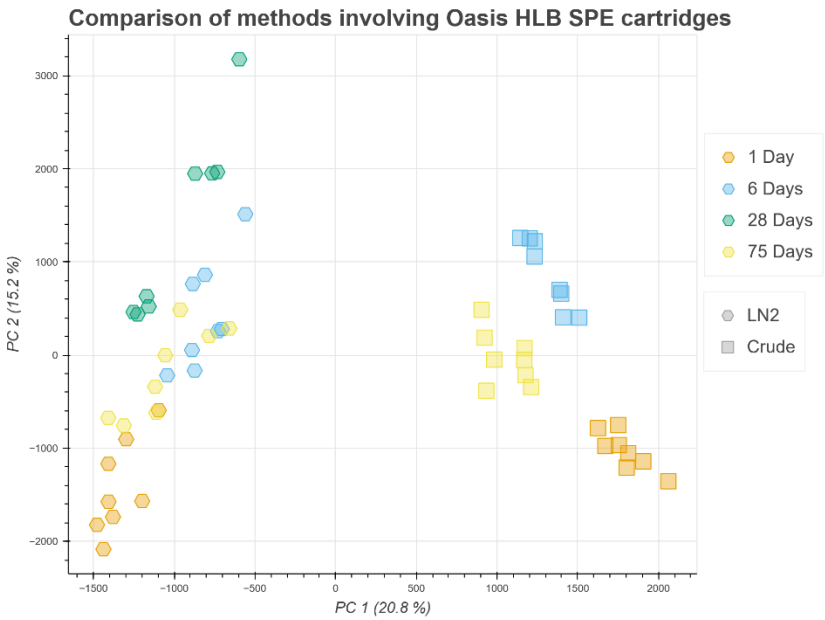

Figure 6: *Principal component analysis of samples stored on an SPE cartridge (CE-OA, squares) and samples prepared by SPE and dried down for storage (FE-SPE, hexagons). Samples stored at 30 °C are not included.*

4. Discussion

4.1. Liquid-liquid extraction – extract and leaf storage

Storage of samples after an LLE without shock-freezing of the leaf tissue showed promising results during the broad screening. All samples from the 7- and 30-day timepoints that were stored at reduced temperatures were tightly grouped together on the PCA (**Figure S10**). The samples stored at 4 °C and -20 °C showed a comparable metabolite profile, which led us to study the LLE approaches in more detail. During the narrow screening we could verify the minimal impact of storage in a freezer compared to refrigerator and obtained a highly reproducible metabolite profiles for both conditions. Overall, our on-site extraction procedure results in samples which more closely represent the metabolite profile of shock-frozen leaf tissue compared to air-dried leaf storage. Even when including the samples stored at room temperature, the profile is closer to our goal than air-dried samples, but there is a notable change depending on storage duration. As such, the storage duration of each sample would become an important factor to control for, which may not be required when storing the extracts at reduced temperatures.

Figure 5C and **D** highlight the extract stability over storage duration, and notably a lower overall change in the metabolite profile than storage of shock-frozen leaf tissue. One reason may be that for on-site LLE, all leaf material is collected into tubes that already contain 200 µL of the extraction solution, which consists of two thirds MeOH and one third water. MeOH has been shown to quench enzymatic activity and is frequently used before metabolite extraction from microbial extracts (Faijes *et al.*, 2007; Link *et al.*, 2008). We thus hypothesize that the immediate contact with MeOH assists with quenching of enzymatic activity for leaf tissue, not unlike flash-freezing with liquid nitrogen. The stability of the MeOH-immersed leaf tissue then becomes relatively independent of temperature and handling. Drying leaf tissue for storage and transport does not have such a quenching step after collection and drying takes more time than flash-freezing or penetration of leaf discs by MeOH solution. Similar effects have been

described previously (Maier *et al.*, 2010) and the instantaneous contact to the solvent seems to be a common theme to assure sample reproducibility.

4.2. SPE as a potential candidate for lower polarity metabolites

We found that storage of extracts on SPE cartridges seemed to result in reproducible metabolite profiles across storage times and conditions, albeit with lower replication than for the other methods tested in broad screening, due to material shortages at the time the work was conducted. As outlined before, SPE shows a significant difference in the metabolite profile compared to LLE and thus may be better suited for research focussing on lower polarity compounds. As our aim was to find a method that can be applied for field studies, the additional logistical challenge of operating a vacuum pump to load the extract onto an SPE cartridge was deemed too large of a hurdle and we proceeded with a focus on LLE-based approaches instead. Besides the operation of a vacuum system, an additional downside is the increased material cost and logistics, which we estimate to at least double the cost per sample.

4.3. Feasibility for field studies

While there are well established procedures for metabolomics sample handling under controlled conditions – most relying on shock freezing in liquid nitrogen followed by uninterrupted cooling to -80 °C – this approach is challenging to apply in field studies. A commonly used approach is to dry the plant tissue, which allows for reproducible results without any cooling; however, the metabolite profile is significantly impacted by the drying process, as shown by the significant separation along PC1 in **Figure 5B**. Our proposed on-site LLE protocol, where a liquid extract is stored in commercial refrigerators, can help fill the gap between shock-frozen and dried leaf extracts. The sample extraction requires some low-cost laboratory chemicals and consumables and almost no infrastructure. Access to electricity is required for the drill for leaf homogenisation (at least to charge a battery) and a refrigerator allows for sample storage over at least two months with minimal shifts in the metabolite profile. While these requirements entail greater logistical challenges than the commonly used dried

plant material method, avoiding the drying process can be worthwhile, especially if more labile metabolites are a focus of the study.

4.4. Limitations of the proposed approach

While the on-site liquid-liquid sample extraction is feasible under logistically challenging conditions and provides samples which more closely represent the metabolite profile obtained from shock-frozen leaves than air-dried leaf storage, it comes with various limitations to consider before using it in large-scale field studies. Extracting metabolites on-site is a time-consuming task which requires some practice before employment in the field. Especially the tissue homogenisation can lead to significant variation between samples until a certain level of practice is reached. Since the exact degree of homogenisation is challenging to standardise, it is also ideally done by one person only to avoid person-to-person variations.

The main limitation is that none of the evaluated methods was able to fully reproduce the metabolite profile obtained from shock-frozen leaf tissue with immediate sample processing. Any storage period did introduce significant shifts in the metabolite profile, even storing shock-frozen leaves at -20 °C. Whether the shifts of the metabolite profile are relevant for a specific application depends on the exact compounds of interest and cannot be generalised here. Lastly, the on-site LLE method for sample collection and extraction was thoroughly tested on maize plants, but no other species was used during this study. Since specialised metabolites of other plants can show a different degradation behaviour, the procedure might not be suitable for all plant metabolomics studies.

5. Supplementary Material

SI1: Detailed description of the extraction procedures

SI2: Detailed description of the data evaluation procedure

SI3: Python script for plotting of PCA data

SI4: Additional pictures and graph

- Figures S1 and S2: Effects of pareto scaling on the LLE optimisation dataset.
- Figure S3: Signal of the three internal standard candidates depending on storage time.

- Figure S4: Photos of the on-site leaf homogenisation and extraction procedure
- Figures S5 – S13: Additional PCA plots of the broad screening.
- Figures S14 – S18: Additional PCA plots of the LLE optimisation.

SI5: R script and output of MANOVA and Pairwise comparison

6. Acknowledgments

We thank Marco Landis and other staff members of the Strickhof (Lindau, Eschikon, Switzerland) for allowing us to collect samples in their maize fields. We thank Urs Stalder and Karoline Rehm for support with maintenance and method development on the UHPLC-MS system.

7. Author Contributions

Conceptualization: JL, MCS; Data Curation: JL; Formal Analysis: JL, SER; Funding acquisition: LB, MCS; Investigation: JL, SER, MS, MCS; Methodology: JL, MCS; Project administration: JL, MCS; Resources: LB, MCS; Software JL, SER; Supervision: LB, MCS; Validation: JL, MCS; Visualization: JL; Writing – original draft: JL, SER, MCS; Writing – review & editing: all authors.

8. Conflict of interest

No conflict of interest declared.

9. Funding

This work was supported by the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme under the grant agreement UPSCALE (SFS-35-2019-2020).

10. Data availability

Processed peak tables will be made available on Dryad upon manuscript acceptance.

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