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Joint GC–MS and LC–MS platforms for comprehensive plant metabolomics: Repeatability and sample pre-treatment

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ABSTRACT

Metabolomics nowadays mostly comprises the application of both LC–MS and GC–MS based approaches. Here we investigate different extraction set-ups for these two established analytical platforms in the field of plant metabolomics. Six extraction approaches for *Arabidopsis thaliana* leaves, varying in extraction solvent composition, extraction temperature and order of solvent addition within the extraction sequence, were analyzed on the two platforms. Our aim was to establish the most suitable analysis protocol, practicable for both LC–MS and GC–MS analysis, in order to obtain as extensive as possible metabolome coverage. One single sample preparation procedure would save time and valuable sample while still offering the complementary datasets generated by GC–MS and LC–MS. All extraction approaches were evaluated based on the following criteria: number of detected *m*/*z*-retention time pairs, heat maps of the detected peaks, and residual enzymatic activity of invertase and phosphatase in the plant leaf extracts. Unsupervised principal component analysis (PCA) was used to evaluate grouping trends between the different extraction approaches. Quality controls, a blend of aliquots of the different extracts, were used to establish a paired evaluation of the repeatability performance of the GC–MS and LC–MS analysis. We conclude that the use of chloroform in the extraction solvent is counterproductive in an untargeted LC–MS metabolomics approach as is heating. Below room temperature (instead of heated) extraction does not significantly degrade GC–MS performance but one should be more cautious with respect to residual enzymatic activity in the plant extract.

1. Introduction

Metabolomics plant analyses aim at the simultaneous detection of all metabolites in plant tissues. While GC–MS is mainly suited for compound classes appearing mainly in the primary metabolism, i.e. amino acids, fatty acids, carbohydrates and organic acids [1], LC–MS is more practicable towards the overall biochemical richness of plants. The latter technique analytically covers the large (semi-polar) group of plant secondary metabolites such as alkaloids, saponins, phenolic acids, phenylpropanoids, flavonoids, glucosinolates, polyamines and derivatives thereof; next to various primary metabolites depending on the type of stationary phase used [2]. Nevertheless, as no single analytical technique is entirely competent in covering the broad metabolic picture, combining multiparallel technologies in metabolomics applications has become indispensable [3], thus aiming at a comprehensive metabolome coverage.

Apart from the analytical platform used, sample preparation too has a vital contribution in defining the array of metabolite classes covered. Any sample preparation protocol in a metabolomics perspective is a compromise between complete recovery of (ultimately) all compound classes, avoiding chemical or physical breakdown of labile metabolites as well as enzyme mediated metabolite conversions, and producing a sample compatible to the separation technique to be used [4]. Sample preparation most of the time starts with immediately quenching metabolism by flash-freezing fresh plant tissues in liquid nitrogen [2,5,6]. Other techniques are freeze clamping or acidic treatment of plant material, although the latter can result in a severe reduction in the number of detectable metabolites [7]. Freeze-drying of samples can also be performed. However, extraction of frozen tissues that still contain the original amount of water may prove more advantageous when compared to extracting freeze-dried samples. Indeed, freeze-drying may potentially lead to the irreversible adsorption of metabolites on cell walls and membranes [8]. Conversely, others promote the use of freeze-drying due to the variable water con-

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tent in fresh plants, causing lower extraction reproducibility and enhanced degradation of metabolites [9]. No matter how, thawing of plant material must be avoided as long as proteins are not fully precipitated. At ambient temperatures, some enzymes such as hydrolases or phosphatases tend to remain or become active even in methanol extraction solutions [10]. An interesting criterion for evaluating residual enzymatic activity in plant biomass is consequently monitoring the degradation of sucrose to glucose and fructose by invertase [1] and the degradation of glucose-6-phosphate and fructose-6-phosphate to phosphate by phosphatase [11]. If enzymatic activity is not immediately stopped during sample preparation, these substrates will disappear. Clearly, a successful metabolomics analysis protocol consists of much more than mere LC–MS, GC–MS or NMR measurements and subsequent data treatment.

In a recent publication, we already focussed on the optimization of pre-LC–MS procedures [12]. The current study extends the search for a comprehensive homogenization/extraction set-up as described in this previous work, hereby focussing on sample stability and expanding the pre-LC–MS procedure to provide compatibility with parallel GC–MS analysis. Having one single sample preparation procedure for both GC–MS and LC–MS saves valuable time and sample, especially taking into consideration that present day metabolomics studies have evolved from a couple of samples to numerous samples in each treatment group. In that respect sample stability is also a vital aspect. Several alternative approaches in restraining the enzymatic activity in harvested *Arabidopsis thaliana* plant material during sample treatment are compared in this study, including the use of chloroform instead of methanol for precipitating proteins and the influence of temperature during extraction on this process (4 °C versus 70 °C). As the order in which the different extraction solvents are added also proves to be crucial [1], the initial addition of a two-phase system to the frozen plant material (e.g., methanol and water together) is compared with successively adding each solvent during the extraction procedure. All investigated procedures were aimed at obtaining a comprehensive plant leaf extract for subsequent GC–MS or LC–MS analysis. Performance evaluation of the different sample treatment approaches was based mainly on number of extracted mass-retention time (*m*/*z*-tR) pairs, heat maps of the detected peaks, and the residual activity of invertase and phosphatase. Unsupervised principal component analysis (PCA) was used to evaluate grouping trends between the different extraction approaches.

Finally, the analytical set-up was designed to provide an indication of repeatability performance of two conventional tools in metabolomics research, i.e. electron-impact gas chromatography mass spectrometry (EI GC–MS) and micro-LC electrospray ionisation quadrupole time-of-flight mass spectrometry (LC–ESI QTOF MS). Quality controls, as described by Sangster et al. [13], were analyzed regularly throughout both analytical runs in an effort to provide proof of the validity of a contiguous set of metabolomics experimental analyses. For target compound analysis, the FDA recommends a coefficient of variation (CV) of 15% regarding the analytical variability (except for concentrations close to the detection limit (LOQ) where a CV of 20% is acceptable). Although metabolomics is of a whole different fundamental analytical nature, the FDA guidance is used as a benchmark towards the repeatability evaluation of both metabolomics approaches.

2. Experimental

2.1. Chemicals

Ribitol, leucine-enkephalin, methoxyamine hydrochloride, pyridine, N-methyl-N-(trimethylsilyl)-trifluoroacetamide, and C_{12}, C_{15}, C_{19}, C_{22}, C_{28}, C_{32}, C_{36} n-alkanes were purchased from Sigma–Aldrich (Bornem, Belgium). Chloroform HPLC grade, acetonitrile and methanol LC–MS grade were supplied by Biosolve B.V. (Valkenswaard, The Netherlands). Formic acid was obtained from Acros Organics (New Jersey, USA). A Synergy 185 system (Millipore Corporation, Bedford, MA, USA) was used to generate high purity water for the preparation of all aqueous solutions.

2.2. Plant growth and extraction of the biological matrix

Plants were grown in the Flanders Institute for Biotechnology (VIB, Department of Plant Systems Biology, Technologiepark 927, Ghent, Belgium). Seeds of *A. thaliana* Columbia-O were sown on a 0.5× MS growth medium. After sowing, media were conserved two days at 4 °C, after which they were placed in a temperature control room with the following conditions: a light intensity of ±350 lx from 6 a.m. to 10 p.m., a relative humidity of 50% and a temperature of 21 °C. Fresh plant leaves were harvested with a pair of micro-scissors and immediately frozen in liquid N_2. Homogenization was performed with mortar and pestle in liquid N_2, after which 150 ± 5 mg of pooled homogenized plant material was weighed in an Eppendorf tube. Each extraction procedure was performed in triplicate on the same pool of plant leaves. The extraction solvent combinations, comprising 300 μl of liquid for each extraction, were spiked in advance with ribitol (internal standard, GC–MS) obtaining a concentration of 180 μg/ml. Extraction procedures A–C are based on liquid extraction with MeOH/H_2O:

(A) Plant material was extracted with 300 μl cold MeOH/H_2O 80/20 (v/v) in a Thermomixer (Eppendorf AG, Hamburg, Germany) during 15 min (1250 rpm, 4 °C) [12].
(B) 240 μl of cold MeOH was added to the plant material containing Eppendorf tube, after which the samples were extracted in a Thermomixer (15′, 1250 rpm, 4 °C). 60 μl of H_2O was added subsequently and samples were mixed again (5′, 1250 rpm, 4 °C).
(C) Identical as procedure B, except that the Thermomixer temperature was set at 70 °C during extraction with MeOH. After 1 min of incubation, the eppendorf tubes are opened for a short moment to relieve built-up gas pressure. The vials remain closed for the rest of the incubation. Immediately after the incubation, all samples are cooled down to 4 °C. As such, all gaseous solvent is liquefied again through condensation.

Extraction procedures D–F are based on liquid extraction with a one-phase $CHCl_3$/MeOH/H_2O mixture:

(D) Plant material was extracted with 300 μl cold $CHCl_3$/MeOH/H_2O 20/60/20 (v/v) in a Thermomixer (15′, 1250 rpm, 4 °C).
(E) 60 μl of cold $CHCl_3$ was added to the plant material containing Eppendorf tube, after which the plant samples were extracted in a Thermomixer (5′, 1250 rpm, 4 °C). 240 μl of MeOH/H_2O 60/20 (v/v) was added subsequently and samples were placed in the Thermomixer again (15′, 1250 rpm, 4 °C) [1].
(F) 180 μl of cold MeOH was added to the plant material, after which the samples were placed in a Thermomixer for 15 min (1250 rpm, 70 °C, with relief of build-up pressure, see above). 60 μl of $CHCl_3$ was then added and samples were mixed again (5′, 1250 rpm, 4 °C). Finally, 60 μl of H_2O was added to the Eppendorf tubes. This method was adopted from the Golm Metabolome Database (http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/analytic/gmd_prot.html).

All extracts were finally sonicated for 5 min (Bransonic Ultrasonic Cleaner 1210, Danbury, CT, USA) and centrifuged (Sigma 3-18K, Sartorius AG, Göttingen, Germany) for 15 min (4 °C, 15,000 rpm). The supernatant (300 μl) was isolated and used for

subsequent LC–MS analysis. For GC–MS analysis, a 50 μl aliquot of the metabolite sample supernatant was further derivatized by methoxyamination, using a 20 mg/ml solution of methoxyamine hydrochloride in pyridine, and subsequent trimethylsilylation with N-methyl-N-(trimethylsilyl)-trifluoroacetamide [14,15]. A C_{12}, C_{15}, C_{19}, C_{22}, C_{28}, C_{32}, and C_{36} n-alkanes mixture was used for the determination of retention time indices [16].

For the quality control (QC) samples, an aliquot (40 μl) of all prepared sample extracts, regardless of extraction procedure, was mixed in an Eppendorf tube in cold conditions. Due to visible contamination, extract D3 was excluded from both the QC preparation and the subsequent LC–MS and GC–MS analysis. The quality control pool was subsequently divided over several vials and analyzed regularly throughout the whole analysis batch, in both GC–MS and LC–MS. The quality control approach for GC–MS and LC–MS metabolomics has been adopted from Sangster et al. [13]. All extracts and QCs were analyzed in parallel on both the LC–MS and GC–MS platform in a 1-day time window.

2.3. Liquid chromatography–mass spectrometry

For the liquid chromatography part, an Alliance 2690 LC system (Waters, Milford, MA, USA) was used. The LC column used was an Atlantis dC18 column 2.1 mm × 150 mm; 3 μm (Waters, Milford, MA, USA). The LC mobile phase consisted of (A) water containing 0.1% (v/v) formic acid; (B) 90/10 acetonitrile/water containing 0.1% (v/v) formic acid. Both eluents (A) and (B) were filtered through a 0.45 μm membrane filter (Alltech Associates, Inc., Lokeren, Belgium) and degassed for 5 min in an ultrasonic bath (Branson, Danbury, CT, USA) prior to use. Gradient elution chromatography was always performed starting with 100% solvent A. Within a 20 min time interval, % B composition was increased to 40%, followed by a %B increase up to 100% within 5 min. This composition was then maintained for 5 final minutes after which the whole system was allowed to re-equilibrate at initial conditions. This generic gradient had separately been optimized [17].

MS experiments were performed using a Q-TOF micro quadrupole orthogonal acceleration time-of-flight mass spectrometer (Waters, Milford, MA, USA) equipped with a dual sprayer orthogonal electrospray source (Z-spray®, LockSpray®). The instrument was operated in positive ion electrospray mode. ESI capillary voltage was optimized to 3000 V and cone voltage was set to 30 V. Full scan spectra were acquired over an m/z 100–1000 range at a scan accumulation rate of 2 scan/s and an interscan delay of 0.1 s. All spectra were collected in continuum, single MS mode. Leucine-enkephalin was used as the lockmass solution (m/z 556.2771; 2 μg/ml in 50/50 AcN/H_2O) and infused at a constant flow rate of 5 μl/min using a Gilson 307 pump (Gilson, Middleton, WI), equipped with a LC Packings flow splitter (Dionex Corporation, Sunnyvale, CA, USA).

2.4. Gas chromatography–mass spectrometry

Samples were analyzed using an Agilent 7683B Series Injector (Agilent, Santa Clara, CA) coupled to an Agilent HP6890 Series gas chromatograph system and a 5973 Mass Selective Detector (Agilent, Santa Clara, CA), i.e. a quadrupole type GC–MS system. A Varian factorFOUR capillary column VF-5 ms (5% phenyl 95% dimethylpolysiloxane, 30 m × 0.25 mm ID, df = 0.25 μm) connected to a 10 m EZ-guard column was used. A constant column flow of 1 ml/min helium was applied. The injector was kept at 230 °C. Samples were splitless injected (1 μl) during 1.5 min using a total flow of 39 ml/min which was reduced to 24 ml/min after 2 min. The temperature programmed separation started at 70 °C for 5 min, and then ramped by 5 °C/min to 325 °C within 51 min. After 1 min at 325 °C, the oven was cooled to the initial temperature of 70 °C within 5.10 min. A temperature equilibration phase of 5 min was allowed before the next injection. The transferline and EI source temperature were 250 and 200 °C. EI spectra were acquired between 60 and 600 Da. The electron multiplier voltage was set on 1700 V.

2.5. Data handling

Raw LC–MS data were processed using QuanLynx® and MarkerLynx® (Waters, Milford, MA), a data processing tool for metabolomics applications. MarkerLynx uses ApexTrack© peak integration to detect chromatographic peaks. The track peak parameters were set as follows: peak width at 5% height 20 s, automatic peak-to-peak baseline noise, intensity threshold 50, mass window 0.5 amu, retention time window 1.0 min, noise elimination level 4.0 and mass tolerance 0.10 amu. The XCMS package [18] in R version 2.6.1 was applied to align the GC–MS chromatograms with the following argument values: xcmsSet (fwhm = 7.5, max = 300, snthresh = 2, step = 0.1, steps = 2, mzdiff = 0.5), group (bw = 7.5, max = 300). All GC–MS data were further normalized using ribitol as an internal standard. AMDIS [19] was used to identify compounds out of the GC–MS chromatograms. Quantifier ions for all compounds (e.g., substrates and end products of invertase and phosphatase) were adopted from the Golm Metabolome Database (http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/msri/gmd_smq.html). SIMCA-P (Umetrics, Umea, Sweden) was used for some aspects of the multivariate data processing. All acquired raw data were subjected to mean-centering, normalization and pareto scaling before multivariate analysis.

3. Results and discussion

3.1. Quality comparison of the different extraction procedures

To evaluate and compare quality, a quality control sample was analyzed regularly throughout both the LC–MS and GC–MS analysis sequences. This sample is prepared from aliquots of all extracts, providing a representative "mean" of the investigated samples. It is not to contain any variability originating from the extraction step and can thus be considered as a mass spectrometric replicate. As a result, monitoring this kind of QC data may be used to evaluate the analytical performance during the analysis sequence on both mass spectrometric tools [20]. Fig. 1 displays the first principal component *t1* of the principal component analysis for all samples versus the samples in run order. The QC runs are included, identified as squares. This principal component is a newly calculated latent variable that explains as much of the present variation as possible in the original dataset. It accounted for 55.1% and 47.0% of the total variability in the GC–MS and LC–MS dataset, respectively. The QCs clearly showed limited variation throughout both analysis approaches. We thus conclude that both analysis methods provide measurement stability for the duration of the analysis sequence. Consequently, the differences observed between the various extraction approaches are to be ascribed to variations in the extraction set-up and not to the variability inherent to the analytical platform. Fig. 1 also clearly indicates extract B3 as an outlier in the GC–MS analysis.

PCA on respectively the GC–MS and the LC–MS data was conducted to study the differences or trends between all extraction procedures (Fig. 2 for LC–MS and Fig. 3 for GC–MS). Strong outliers (samples outside the Hotelling's T^2 95% tolerance ellipse) were excluded from further data processing (i.e. sample B3 for GC–MS analysis). The PCA score plot in Fig. 2 clearly distinguishes three groups within the procedures as analyzed by LC–MS: the extrac-

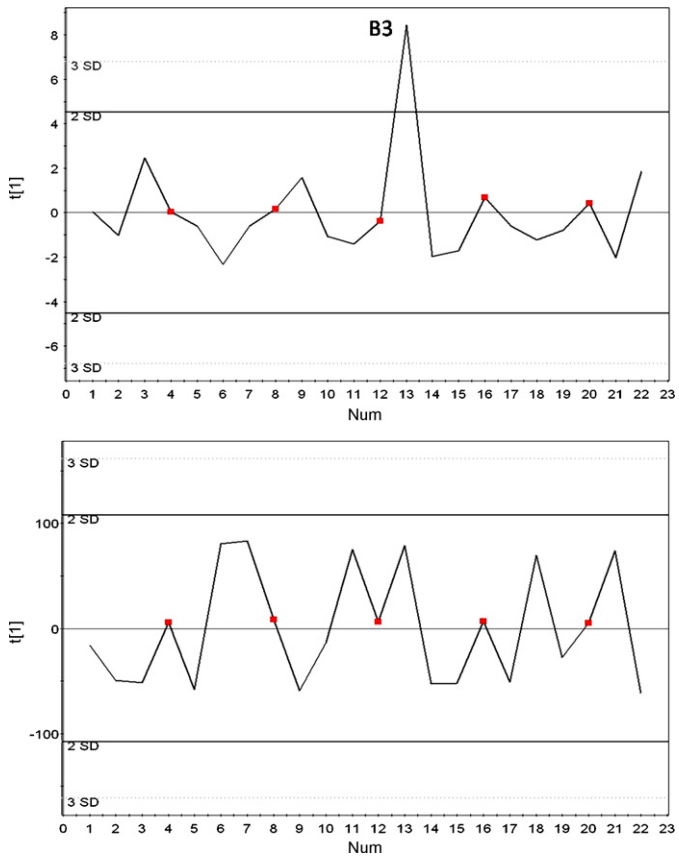

Fig. 1. Time series plot of the first PCA component for GC–MS analysis (upper pane) and LC–MS analysis (lower pane). The 2σ and 3σ limits are also shown. QCs are shown in squares. Extract B3 is spotted as an outlier in the GC–MS analysis.

tion step performed with 80/20 methanol/H_2O without heating of the Thermomixer (procedures A and B), the extraction step performed with 20/60/20 chloroform/methanol/H_2O without heating of the Thermomixer (procedures D and E) and finally the extraction procedures with the Thermomixer heated up to 70 °C (procedures C and F). Not only do the procedures including heating during extraction separate themselves form the other procedures, heating moreover clearly increases the analytical variability. The samples of procedures C and F do show a larger scatter in the PCA score plot compared to the other procedures. PCA also separates the different approaches when using the GC–MS data (Fig. 3), albeit less conclusive. In addition, procedures B and D cannot be distinguished based on those metabolites detected in the GC–MS analysis. The corresponding loadings plots allow pinning down those metabolites responsible for the classification of the extraction procedures. In a metabolomics application, identification of this relevant subset of metabolites is the next step. The reliable identification of metabolites using GC–MS libraries is a major advantage within this platform. On the contrary, identification still is the bottleneck in current LC–MS analysis, as no comprehensive metabolite libraries are available for this metabolomics platform. In spite of this, some metabolites could be annotated using accurate mass measurement with a LockSpray® device [17] and subsequent database search (see Fig. 2).

If, at the other hand, we compare the number of detected *m*/*z*-tR pairs between the different procedures for the LC–MS analysis (Fig. 4), it is clear that procedure A produces the highest number of *m*/*z*-tR pairs (3773; $n = 3$). Extraction with the three-phase system (i.e. chloroform/methanol/H_2O or procedure D, E and F) yields a significantly reduced number of *m*/*z*-retention time pairs compared with the two-phase system (i.e. methanol/H_2O or procedure A, B and C). As an example, the observed difference in extracted signals between procedure A and D amounts up to 43% (3773 versus 2144 *m*/*z*-tR signals). Heat maps, plotting LC–MS *m*/*z*-value against retention time, unravel clear regions of contrast between the different extraction procedures, emphasizing this observation (Fig. 5). The retention time zone ranging from 17 to 27 min shows the largest between profile dissimilarity. The procedures based on extraction with methanol/H_2O (procedures A, B and C) show a higher density profile within this region compared to the extractions based on chloroform/methanol/H_2O (procedures D, E and F). Clearly, the addition of $CHCl_3$ to the extraction solvent has a significant influence on the solubility of the secondary metabolites eluting in the time range from 17 to 27 min. Heating up to 70 °C is obviously also less favourable (LC–MS analysis results), independently of the extraction solvent combination used. Procedure C, e.g., detects 29% less *m*/*z*-tR pairs than procedure A, although the same extraction solvent combination is used (3773 versus 2687 *m*/*z*-tR signals). Extraction procedures using a heated Thermomixer at 70 °C (pro-

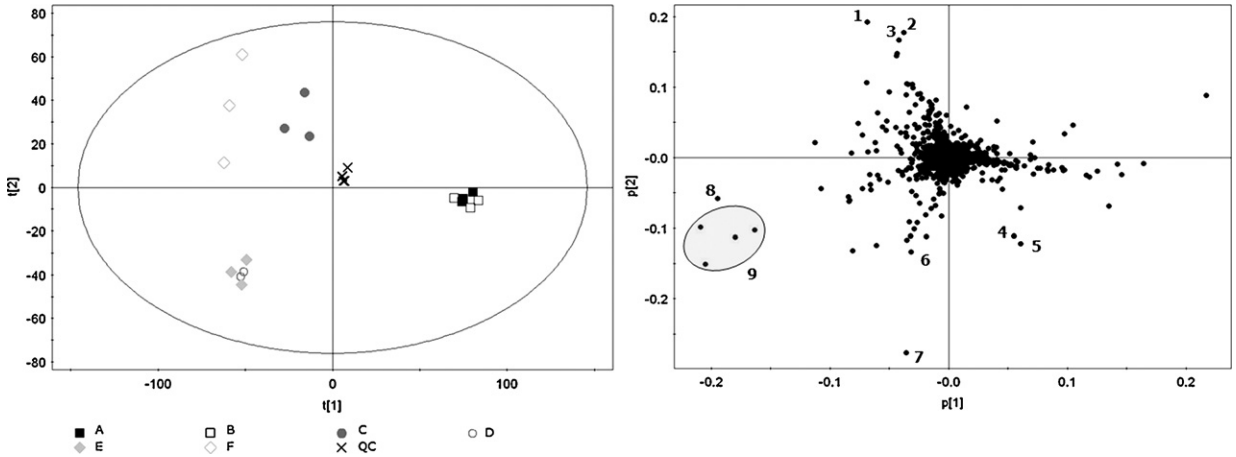

Fig. 2. Score (left) and loadings plot (right) obtained from PCA of the entire LC–MS dataset; *t1* and *t2* of the score plot accounted for 47.0% and 12.8% of the total variability in the pareto scaled data of all extraction procedures, respectively. Some metabolites responsible for the different extraction set-up could be annotated based on accurate mass measurement using lockmass calibration: 1. glutathione (*m*/*z* 308.0907; ppm −2.92); 2. deoxyadenosine (*m*/*z* 252.1078; ppm −7.42); 3. [sinapate − H_2O + H]$^+$ (*m*/*z* 207.0652; ppm −2.37); 4. 1-caffeoyl-4-deoxyquinic acid (*m*/*z* 339.1072; ppm −2.30); 5. [1-caffeoyl-4-deoxyquinic acid − H_2O + H]$^+$ (*m*/*z* 321.0965; ppm −2.80); 6. adenosine (*m*/*z* 268.1038; ppm −3.13); 7. 5-methylsufinylpentyl nitrile (*m*/*z* 146.0633; ppm −4.93); 8. [sinapate − H_2O + H]$^+$ (*m*/*z* 207.0657; ppm −2.41); 9. Background ions originating from the purified water in eluent A.

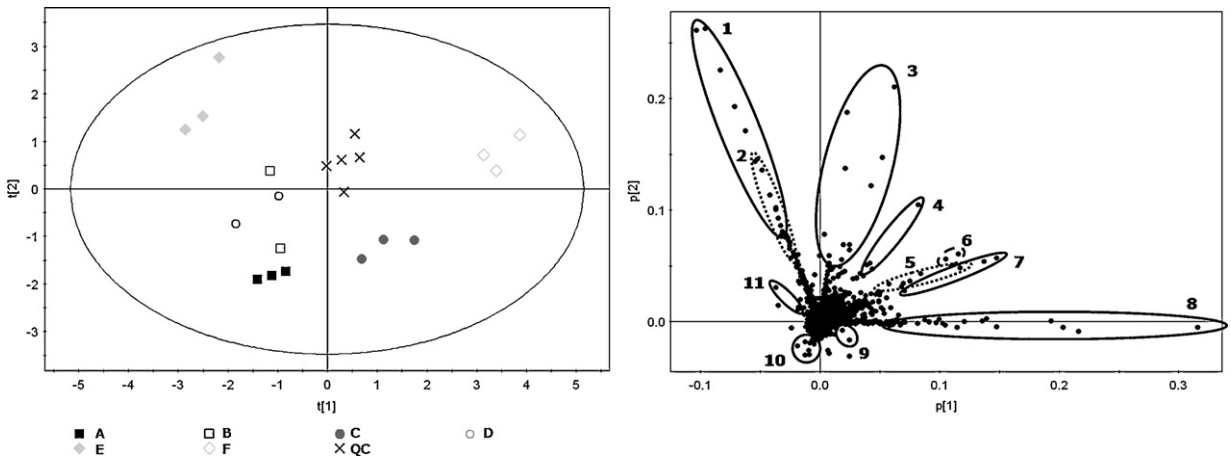

Fig. 3. Score (left) and loadings plot (right) obtained from PCA of the entire GC–MS dataset; *t1* and *t2* of the score plot accounted for 51.0% and 23.0% of the total variability in the pareto scaled data of all extraction procedures, respectively. The metabolites most responsible for the classification of the different extraction set-ups are marked: 1. glucose; 2. galactose; 3. fructose; 4. phosphoric acid; 5. myo-inositol; 6. ethylglucopyranoside; 7. pyroglutamic acid; 8. sucrose; 9. galactonic acid; 10. glycerol; 11. glycerol-3-phosphate.

cedures C and F) show an overall less dense heat map compared to the cold extraction procedures. Five of the most intense ions in the region from 17 to 27 min were extracted and their peak areas were compared between the different extraction approaches as a proof of concept (Fig. 6). Addition of $CHCl_3$ clearly decreases the solubility of these metabolites in the extraction solvent, whereas heating to 70 °C during extraction removes substances from the final extract most probably because of enhanced chemical degradation. Several authors discussed the temperature sensitivity of phenolic compounds already. Temperatures above 40 °C resulted in a decreased extraction yield of polyphenolic compounds due to degradation, caused by hydrolysis, internal redox reactions and polymerisations [21–24]. Because the secondary metabolites eluting in the region from 17 to 27 min are missed in the extractions based on $CHCl_3$/MeOH/H_2O 20/60/20 (v/v/v), background ions originating from the purified water in eluent A show up more pronounced in these mass chromatograms. This explains the presence of these contaminant peaks in the PCA loadings plot of the LC–MS data in Fig. 2. This presence of the background ions is instrumental in classifying these samples away from the other extraction procedures in the resulting PCA score plot.

The number of masses detected in the GC–MS analysis shows a somewhat opposite tendency compared to the LC–MS analysis. Heating up the Thermomixer to 70 °C (procedures C and F) yields a slightly higher portion of detectable *m*/*z*-tR pairs compared to a reduced temperature extraction (4 °C) using the Thermomixer (differences from minimum 4% to maximum 9% more *m*/*z*-tR pairs). The range of metabolites that is detected in the GC–MS analysis (i.e. mainly primary metabolites) seems to dissolve better when the temperature of the extraction solvent is raised, as in procedure C and F. Opposite to the LC–MS data, heat maps plotting *m*/*z*-value against retention time for the GC–MS data unravel no distinct regions of contrast between the profiles of the different extraction procedures (data not shown). We conclude that in any of the extraction procedures none of the metabolite classes are likely to be missed in the GC–MS analysis. In the light of obtaining an optimized extraction procedure for a comprehensive metabolomics approach on both GC–MS and LC–MS, the number of peaks detected in both platforms was summed up for each extraction procedure (dotted line in Fig. 4). As a result, procedure A and B yield a significantly higher number of metabolites compared to the other extraction set-ups. Overall, procedure A provides the best results in terms of variability and the number of detected *m*/*z*-tR pairs taking GC–MS and LC–MS data together. Aiming for a combined GC–MS and LC–MS approach inevitably entails accepting a compromise. Using procedure A as front-end is least at the expense of overall performance. Nevertheless, the results also indicate that no matter how distinctly a method pursues comprehensiveness in metabolite yield, 100% full metabolite coverage is beyond reach.

It is therefore a judicious choice for every metabolomics experiment whether a more focussed approach thus extraction, guided by the experimental aim, is followed or an untargeted one while aware of its inherent performance limitations.

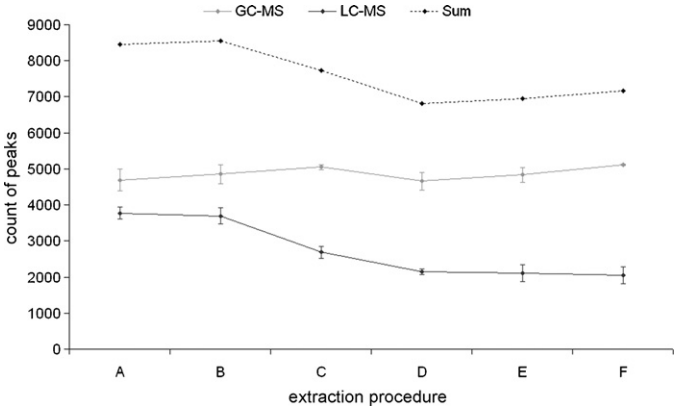

Fig. 4. Count of *m*/*z*-tR pairs or peaks detected in the different extraction approaches in both GC–MS as LC–MS analysis ($n = 3$; $n = 2$ for procedure D). The dotted line represents the sum of *m*/*z*-tR pairs for a specific extraction procedure.

3.2. Enzymatic activity considerations

As the homogenization step is carried out in liquid nitrogen, no enzymatic conversion is likely to occur in this stage of sample treatment. The Thermomixer based extraction using a 2:1 ratio solvent volume to frozen tissue weight is, nevertheless, a critical step regarding enzyme reactivation. As mentioned in Section 1, both invertase and phosphatase activity can be monitored for evaluating residual enzymatic activity in plant extracts [1,11]. Both substrates and end products of these enzymes can be detected using GC–MS analysis (quantifier ions were adopted from http://csbdb.mpimp-golm.mpg.de/csbdb/gmd/msri/gmd_smq.html).

Phosphatase hydrolyses both glucose-6-phosphate (6TMS: *m*/*z* 387.1 at tR 38.2) and fructose-6-phosphate (6TMS: *m*/*z* 315.0 at tR 38.2) with the formation of phosphate (3TMS: *m*/*z* 314.1 at tR

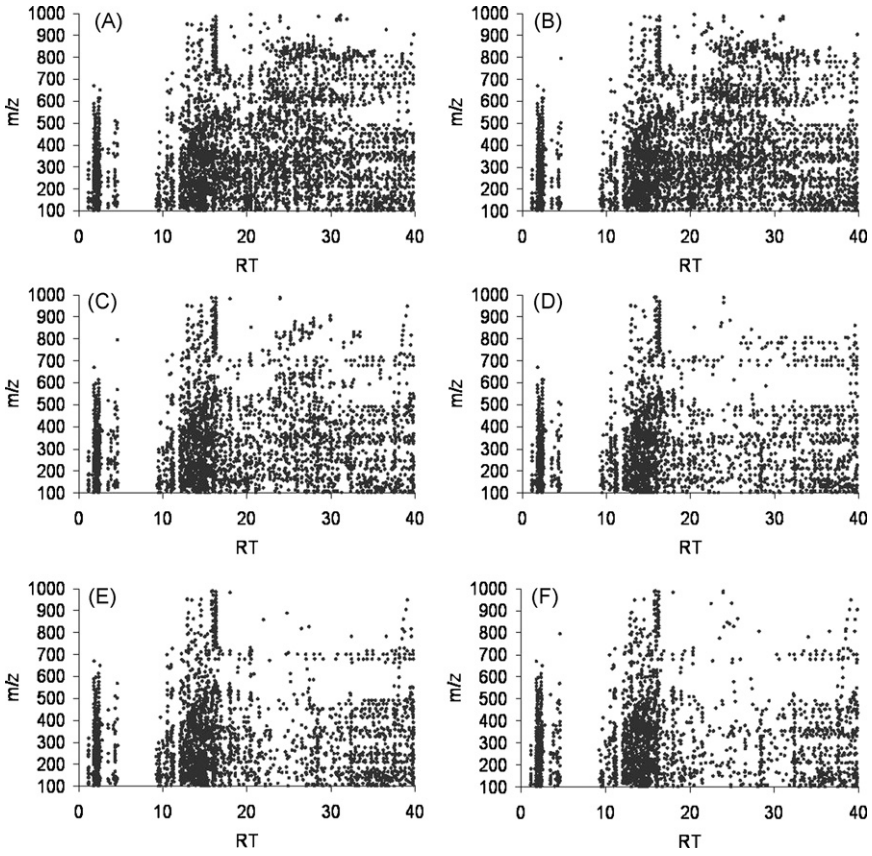

Fig. 5. Heat maps from the LC–MS analyses of all extraction procedure set-ups showing the distribution of m/z with retention time.

16.1). As can be seen in Fig. 7, a high amount of phosphate is present in all plant extracts. The ratio of substrate and end products remains, however, practically equal for all extraction procedures thus indicating that they all perform similarly. The second enzyme that is monitored is invertase, which hydrolyses sucrose (8TMS: m/z 437.3 at tR 43.2) resulting in glucose (5TMS: m/z 160.1 at tR 30.6) and fructose (5TMS: m/z 307.1 at tR 30.2). Fig. 8 outlines the result of the invertase activity evaluation in all plant extracts. The addition of water, separately from the organic solvent (both methanol and chloroform), results in a higher concentration of both glucose and fructose in the particular plant extract. This indicates a higher residual activity of invertase. The mean normalized peak areas for glucose and fructose were 23.89% and 37.39% higher within the methanol/H_2O extraction set-up (procedure B versus procedure A) and 19.75% and 66.63% higher within the chloroform/methanol/H_2O extraction set-up (procedure E versus procedure D), respectively. The degradation of sucrose is significantly lower (i.e. the concentration is higher in the extraction solvent) in the case of a heated extraction (70 °C) as can be deduced from the concentration of sucrose which is 28.98% and 45.71% higher for extraction procedures C and F compared with procedures A and D, respectively. These results can also be deduced from the PCA loadings plot (see Fig. 3) where sucrose is mainly responsible for the classification of both procedures C and F. Consequently, a heated extraction set-up can be considered as superior towards

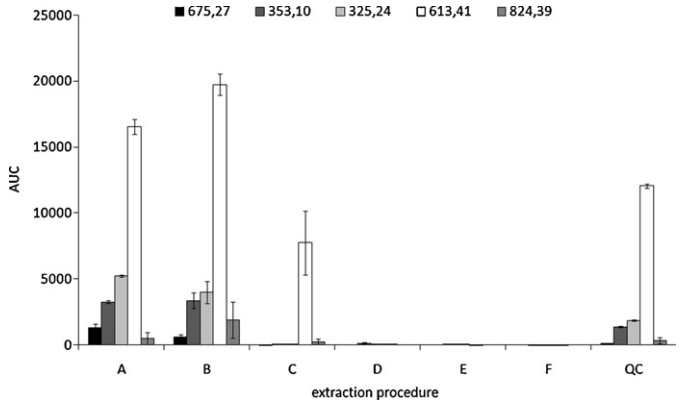

Fig. 6. Plot representing the mean peak area ± S.D. ($n = 3$; for QC $n = 5$) of the five most intense peaks in the retention time region from 17 to 27 min. m/z 675.27 could be annotated as icariin, a flavonoid glycoside (m/z 675.2652; ppm −0.13).

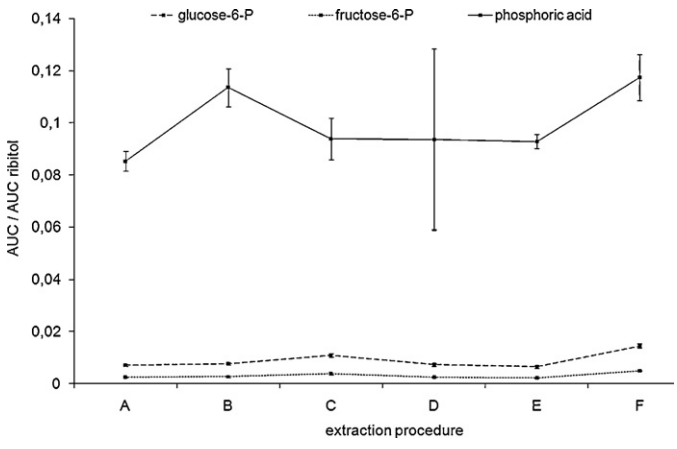

Fig. 7. Plot representing the mean normalized peak area ± S.D. ($n = 3$) of G-6-P (substrate; dashed line), F-6-P (substrate; dotted line) and phosphoric acid (end product; full line) from phosphatase activity in all extraction procedures.

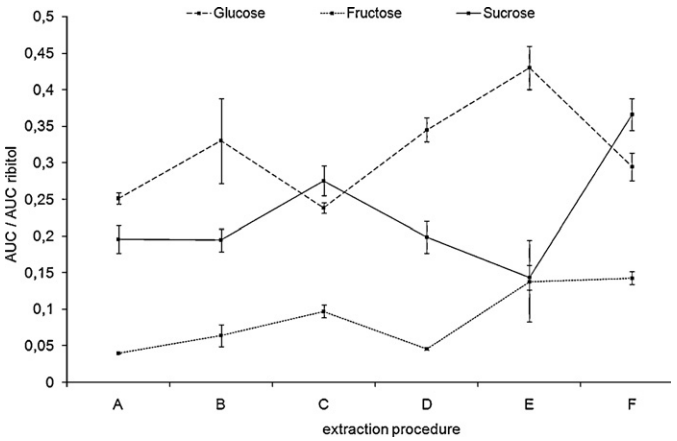

Fig. 8. Plot representing the mean normalized peak area ± S.D. ($n = 3$) of the substrate (sucrose; full line) and the end products (glucose; dashed line, and fructose; dotted line) from invertase activity in all extraction procedures.

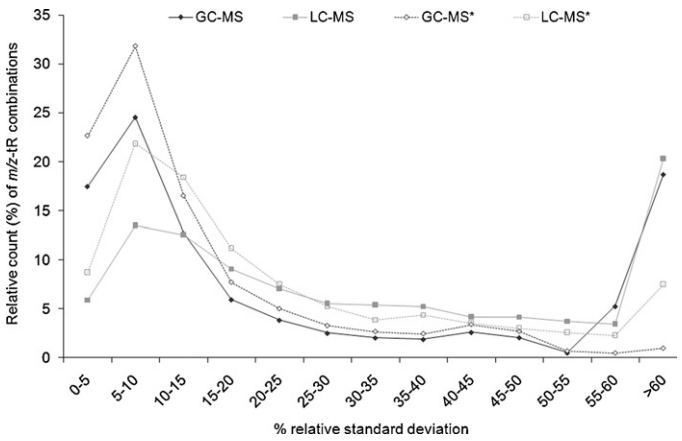

Fig. 9. Relative count (%) of detected m/z-retention time pairs in relation to the repeatability for both GC–MS (normalized using the internal standard ribitol) and LC–MS analysis of the QC samples ($n = 5$).
*Only for the number of peaks that are always detected (i.e. have a positive intensity across all QC samples).

the inhibition of residual enzymatic activity of invertase in plant leaf extracts.

3.3. Repeatability performance of LC–MS versus GC–MS

QCs, being mass spectrometric replicates, were used to quantify the repeatability of both the GC–MS and the LC–MS analysis. Although of a whole different fundamental analytical nature, all of the detected peaks or m/z-tR signals in our metabolomics set-up were evaluated according to the FDA guideline for targeted quantitative analysis. Untargeted analysis is by definition susceptible to more variation. Nevertheless, the 15 or 20% CV FDA thresholds provide at least some kind of benchmark in terms of repeatability requirement, as the latter is not easily found in metabolomics literature. Fig. 9 represents a relative frequency distribution of the repeatability calculated over all detectable metabolites in the QC samples. A distinction is made between all detectable m/z-tR signals in the QC samples (full lines) and the m/z-tR signals always present in all the QCs (dotted lines). As can be seen, the majority of the detected signals are measured with a relative variability below 15%. One can also see that (normalized) GC–MS has an overall better repeatability compared to the LC–MS approach. This is not entirely surprising as the peak areas of the GC–MS data are standardized by the normalization process with the internal standard ribitol. The usefulness of normalization with the internal standard ribitol for GC–MS analysis is corroborated by the frequency distribution for not-normalized GC–MS results, especially in the CV bin from 0 to 5%. Normalized GC–MS results show an increase in the relative frequency for the 0–5% CV bin with 11 percentage points compared with the not-normalized 0–5% CV bin (data not shown). Preliminary work (data not shown) unfortunately proved

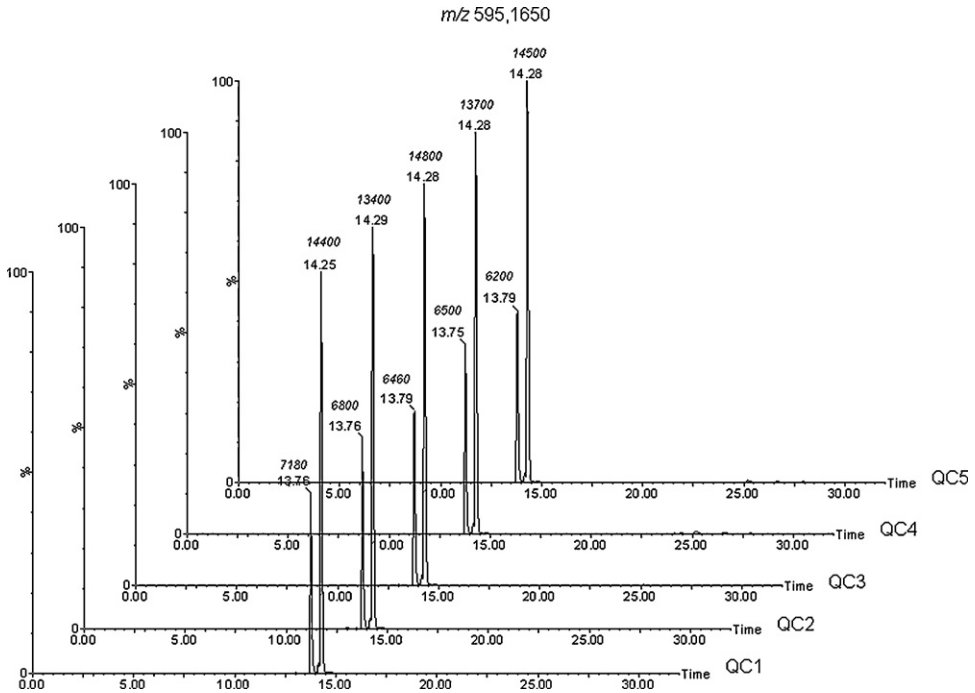

Fig. 10. Extracted ion chromatograms of m/z 595.1650 in 5 QC samples ran in between all plant extract samples demonstrating the repeatability in peak height (indicated in *italics* above peaks) and retention time (indicated above peaks).

Table 1
Percentage of detected *m*/*z*-tR signals in both analytical platforms complying with the FDA target compound analysis criteria.

Analysis platform	*m*/*z*-tR signals considered	Number of *m*/*z*-tR signals	<15%	<20%
GC–MS	All	5544	55%	61%
	Ever present	4276 (77%)	71%	79%
LC–MS	All	3293	32%	41%
	Ever present	1590 (48%)	49%	60%

that normalization does not work in the LC–MS based approach. Nevertheless, if we focus on the peaks that have a positive intensity across all of the QC samples, LC–MS analysis does prove to be a well functioning analysis platform. This again confirms that the peaks of lowest intensity, consequently variable in occurrence throughout all the samples, are prone to the greatest variability, especially in LC–MS analysis [20,25]. Table 1 reports on the percentage of peaks that fulfil the FDA 15% and 20% CV criteria. 32% or 1052 out of the 3293 detected *m*/*z*-tR combinations were acceptable in the LC–MS analysis using the ≤15% CV criterion, while 41% were acceptable at the ≤20% level. Normalized GC–MS analysis reproducibly detected 3033 out of 5544 detected *m*/*z*-tR pairs or 55% within the 15% CV criterion, while 61% were within the 20% level, suggesting a better repeatability of the GC–MS analysis. Table 1 also presents the number of peaks that have a positive intensity across all QC samples, i.e. 1590 of the 3293 *m*/*z*-tR signals or 48% for LC–MS and 4276 of the 5544 *m*/*z*-tR signals or 77% for normalized GC–MS. The amount of ever detected peaks that fulfil the 15% CV criterion was 49% for the LC–MS analysis and 71% for the normalized GC–MS analysis, which increased to respectively 60% and 79% for the 20% CV level. Here the LC–MS analysis slightly catches up with the GC–MS analysis with respect to repeatability.

Fig. 10 depicts retention time and peak height repeatability of a representative ion, *m*/*z* 595.1650, in the successive QC samples, covering a 24 h time span of LC–MS analyses. This ion at tR 14.28 (R.S.D.% tR 0.11%; R.S.D.% peak height 4.14%; $n = 5$) represents kaempferol 3-*O*-glucoside 7-*O*-rhamnoside [26], based on accurate mass measurements, with a mass deviation of −2.18 ppm. The ion also appears at tR 13.79 (R.S.D.% tR 0.14%; R.S.D.% peak height 5.66%; $n = 5$) representing a fragment of a compound with *m*/*z* 741.2220, itself annotated as kaempferol 3-*O*-rhamnosyl glucoside 7-*O*-rhamnoside [26], with a mass deviation of −2.91 ppm. The presence of flavonoids is not uncommon in the plant family of the Brassicacea, to which *A. thaliana* belongs, and many class members could be identified from our datasets based on accurate mass and MS/MS data for selected mass-retention pairs.

4. Conclusions

Conventional MS platforms such as EI GC–MS and micro-LC ESI QTOF MS are frequently used in the field of plant metabolomics. In untargeted metabolomics assays, data repeatability is of the utmost importance. Because no guidelines regarding reproducibility have yet been formulated for metabolomics procedures, our results were evaluated against the well-known FDA target compound analysis criteria. Based on the evaluation of QC samples, 41% of the detected *m*/*z*-tR combinations in the LC–MS analysis showed a repeatability lower than 20%, which increases up to 60% in the case of ever detected peaks. GC–MS analysis, including a normalization step, performed better, with percentages of 71% and 79%, respectively. As a result, these conventional mass spectrometric platforms can be considered to provide good repeatability, thus establishing analytical tools that perform well in an untargeted metabolomics assay.

With respect to the extraction of metabolites from plant leaves, the use of chloroform in the extraction solvent seems counterproductive in an untargeted LC–MS metabolomics approach, as the number of detected *m*/*z*-tR pairs decreases with at least 40% compared to a simple water/methanol mixture. Furthermore, an important set of secondary metabolites, eluting in the retention time range from 17 to 27 min in our set-up, is almost completely lost. A heated extraction at a temperature of 70 °C also decreases the number of features detected using LC–MS, while in GC–MS, heating brings about a slight increase in the number of peaks detected. Nevertheless, in terms of enzymatic inactivation, heating during the extraction performs superior compared to the same extractions in cold conditions. This could especially be concluded from the residual invertase activity in the plant extracts. While our aim was to establish the most suitable analysis protocol in order to obtain an as extensive as possible metabolome coverage, it is clear that there will never be one single ideal way to prepare 100% comprehensive plant extracts. Having compromises is unavoidable in non-targeted metabolomics approaches. For the metabolomics analysis of *A. thaliana* plant leaves a procedure entailing the extraction of 150 mg of homogenized plant leaves with 300 μl cold MeOH/H_4O 80/20 (v/v) in a Thermomixer during 15 min (1250 rpm, 4 °C) achieved the best quality in terms of repeatability, number of extracted metabolites, PCA analysis potential (e.g., informative clustering) and portability between GC–MS and LC–MS. Attention should nevertheless be paid to residual enzymatic activity in the plant extract. Indeed, once an analytical approach chosen, it is important that all confounding factors are known and monitored closely. Repeatability evaluation is vital in that respect. Only then, fluctuations in metabolite levels can be properly translated into biological knowledge.

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