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LC–MS metabolic profiling of *Arabidopsis thaliana* plant leaves and cell cultures: Optimization of pre-LC–MS procedure parameters

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ABSTRACT

This study treats the optimization of methods for homogenizing *Arabidopsis thaliana* plant leaves as well as cell cultures, and extracting their metabolites for metabolomics analysis by conventional liquid chromatography electrospray ionization mass spectrometry (LC–ESI/MS). Absolute recovery, process efficiency and procedure repeatability have been compared between different pre-LC–MS homogenization/extraction procedures through the use of samples fortified before extraction with a range of representative metabolites. Hereby, the magnitude of the matrix effect observed in the ensuing LC–MS based metabolomics analysis was evaluated. Based on relative recovery and repeatability of key metabolites, comprehensiveness of extraction (number of *m*/*z*-retention time pairs) and clean-up potential of the approach (minimum matrix effects), the most appropriate sample pre-treatment was adopted. It combines liquid nitrogen homogenization for plant leaves with thermomixer based extraction using MeOH/H_2O 80/20. As such, an efficient and highly reproducible LC–MS plant metabolomics set-up is achieved, as illustrated by the obtained results for both LC–MS (8.88% ± 5.16 versus 7.05% ± 4.45) and technical variability (12.53% ± 11.21 versus 9.31% ± 6.65) data in a comparative investigation of *A. thaliana* plant leaves and cell cultures, respectively.

1. Introduction

Plant metabolomics can be defined as the technology geared towards providing an essentially unbiased, comprehensive qualitative and (semi-)quantitative overview of the metabolites present in plant tissues, at a certain point in time [1]. Liquid chromatography (LC) coupled to mass spectrometry (MS) offers the best combination of sensitivity and selectivity, and therefore is indispensable in most metabolomic approaches [2]. It covers a wide mass range and targets many compound classes, representing the overall biochemical richness of plants. LC–MS detects 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 [3].

To reduce experimental error for subsequent statistical analysis of different phenotypes, ample attention to the sample preparation approach is a key factor in good metabolomic practice. Metabolomic plant analyses are 'quick-and-dirty' methods that try to be as comprehensive but also as fast as possible, aiming to maximally avoid artefactual alterations of the metabolite pattern. Inevitably this weighs on the potential to define each metabolite in a precise and reproducible way [4]. Postharvest treatment begins with rapidly stopping the inherent enzymatic activity of plant samples, generally by flash-freezing fresh plant tissues immediately in liquid nitrogen [3,5,6]. Before extraction, plant material has to be homogenized first. Various techniques can be used: grinding with a mortar and pestle in liquid nitrogen [3], mixing with an Ultra Turrax device [7] and milling in a vibration mill [3,8,9]. It is important to keep in mind that the degree of homogenization determines the efficiency at which the extraction solvent can penetrate the tissue [9]. In metabolomics, cold extraction is mostly preferred for the sake of stability of the compounds and reproducibility of the analysis. The selection of the extraction solvent hereby greatly influences the range of the detectable metabolites [10]. The chemical diversity of the plant metabolome that encompasses a vast array of compounds differing in polarity, molecular mass and amount present, makes that each extraction solvent composition has unavoidable

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bias towards certain metabolite classes [1]. Given the metabolomics goal, an extraction method should be developed where the number and amount of metabolites extracted is maximized, nevertheless in keeping with reproducible operating procedures [9]. Extraction of plant tissues with aqueous methanol is frequently used in LC–MS metabolomic applications [11–14]. Also, to enhance the range of extractable metabolites, combinations of solvents like water/methanol/chloroform [8,9] or aqueous acetonitrile [10] are often encountered. In addition to solubilising the metabolites, a good extraction solvent should equally prevent physical or chemical alterations of the molecules extracted [15].

Clearly, pre-LC–MS treatment, including homogenization and extraction, of plant samples is feasible in many ways. However, few studies, comparing the performance characteristics of different approaches, have been carried out with respect to finding an optimal pre-LC–MS treatment. In this study, several procedural variations have been compared resulting in a simple method for homogenizing plant tissue and subsequently reproducibly extracting as many metabolites as possible for the analysis of the *Arabidopsis thaliana* metabolome in either tissue homogenate or cell cultures, using conventional reversed-phase micro-liquid chromatography electrospray ionization quadrupole time-of-flight mass spectrometry. Our aim is true metabolomics analysis, i.e. the quantitative analysis of all the low molecular weight molecules present in cells. Consequently, attention has been focussed on: the number of detectable *m*/*z*-retention time pairs, absolute extraction recovery, LC–MS matrix effect, process efficiency, and the influence thereupon of extraction solvent type and extraction solvent volume. Finally, the resulting pre-LC–MS procedure has been implemented in *A. thaliana* cell cultures as well as plant leaves, focussing on the variability aspect within analyses.

2. Experimental

2.1. Chemicals

Metabolites L-leucine, L-phenylalanine, *S*-adenosyl-L-methionine (SAM), adenosine 5′-diphosphoglucose (ADP-glucose), adenine, cytidine, uridine 5′-monophosphate (UMP), zeatin, gibberellic acid (GA3), (±)-jasmonic acid, (±)-*cis*,*trans*-abscisic acid, epibrassinolide, spermidine, *p*-coumaric acid and chorismate were all purchased from Sigma–Aldrich (Bornem, Belgium). D-(+)-lactose and chloroform HPLC grade were purchased from Merck (Darmstadt, Germany). 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. Standards and samples

Stock solutions of 1 mg/ml of all metabolites were prepared in varying methanol/acetonitrile/water mixtures, chosen such as to contain as much organic solvent as possible, but taking into consideration the solubility characteristics of the individual compound. Concentrations of metabolites when spiked to biological samples ranged from 2 to 15 nmol/mg plant tissue.

2.3. Plant and cell growth and extraction of the biological matrices

Plants and cell cultures 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 2 days in 4 °C, after which they were placed in a Temperature Control room with the following conditions: a light intensity of ±350 lux from 6 a.m. to 10 p.m., a relative humidity of 50% and a temperature of 21 °C. Cell suspension cultures of *A. thaliana* ecotype Landsberg erecta were grown in MSMO medium (Sigma, St Louis, MO, USA) supplemented with 3% sucrose. Cells were diluted 10-fold on a weekly basis.

Fresh plant leaves or alternatively, cell cultures were immediately frozen in liquid N_2. Homogenization was always performed on 100 ± 5 mg of frozen plant material. Homogenization/extraction devices and conditions used: (A) ball mill (CAPII, Henry Schein, New York, USA) during 2 min in prechilled capsules; (B) mortar and pestle; (C) Ultra Turrax (IKA werke GmbH & CoKG, Staugen, Germany) during 2 min in liquid N_2 bath; (D) Ultrasonic Disintegrator (MSE, Crawley, Sussex, UK) during 2 min in an ice-bath and (E) Thermomixer (Eppendorf AG, Hamburg, Germany) during 15 min (1250 rpm, 4 °C). Procedures A, B and C were all one-step homogenization-extraction approaches performed with 1 ml of 20/60/20 H_2O/MeOH/$CHCl_3$. Procedures D and E were performed with 200 μl of initially the same solvent mixture, later on using MeOH/H_2O 80/20 (v/v). All extracts were sonicated for 5 min (Bransonic Ultrasonic Cleaner 1210, Danbury, CT, USA) and centrifugated (Sigma 3–18 K, Sartorius AG, Göttingen, Germany) for 15 min (4 °C, 15,000 rpm). Supernatant was isolated and used for LC–MS analysis. Frozen cell culture samples were extracted with 80/20 (v/v) MeOH/H_2O in the Thermomixer (15 min, 1250 rpm, 4 °C). Further treatment was identical to plant leaves extracts.

2.4. Liquid chromatography–mass spectrometry

For the liquid chromatography part, an Alliance 2690 LC system (Waters, Milford, MA, USA) was used. 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 5 min lasting %B increase up to 100%. This composition was then maintained for five final minutes after which the whole system was allowed to re-equilibrate at initial conditions. This generic gradient had separately been optimized [16].

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 both positive and negative ion electrospray mode (separate runs). ESI capillary voltage was optimized to 3000 V and cone voltage was set on 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.

2.5. Data acquisition and handling

The Alliance LC system and Q-TOF micro™ instrument were controlled using the MassLynx® software version 4.0 (Waters, Milford, MA, USA). Raw LC–MS data were further processed using QuanLynx® and MarkerLynx® (Waters, Milford, MA), a data processing tool for metabolomics applications. SIMCA-P (Umetrics,

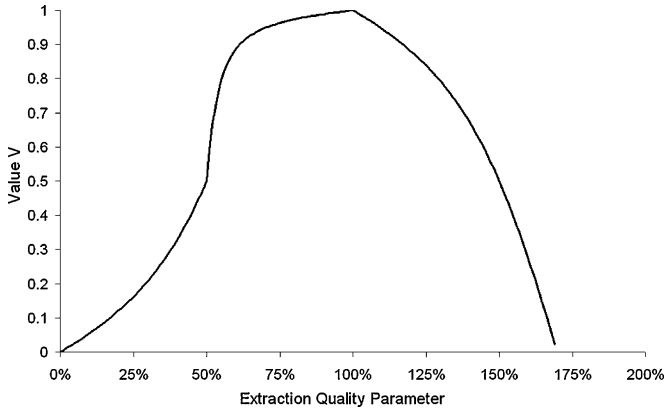

Fig. 1. Transformation function for extraction quality parameter evaluation.

Umea, Sweden) was used for some aspects of the multivariate data processing.

2.6. Transformation function for extraction quality parameters

In order to evaluate the overall (different compounds together) absolute recovery and process efficiency of the different sample preparation procedures, we used a transformation function *F*. As shown in Fig. 1, each individual extraction quality parameter (EQP) for each of the 16 metabolites, as calculated by comparison to a pure metabolite mix, is transformed to a new value, designated *V*, by a particular function *F* [17]. The transformation function has the following characteristics: for "acceptable" EQPs, between 50 and 100%, $y = 1 + w((3/2) - x) + (1/2)\sqrt{1 + 4(w^2 - 1)((3/2) - x)^2}$ (x is EQP and w is a weighing factor = 10), while for EQPs below 50% [$y = (1/2)w(2x - 1) + (1/2)\sqrt{1 + (w^2 - 1)(2x - 1)^2}$, $w = 2$] and above 100% [$y = 1 + w(x - (1/2)) - (1/2)\sqrt{1 + 4(w^2 - 1)(x - (1/2))^2}$, $w = 2$] a more than linear penalty is assigned. All EQPs that extend beyond these limits are assigned the value 0.01. Calculation of the geometric mean of these new *V* values resulted then in the response value, referred to as *R*. Therefore, comparison of all the individual compound's EQPs between particular extraction procedures is now reduced to the comparison of the value *R* for those extraction setups [18].

3. Results and discussion

3.1. Pre-analytical procedure optimization and extraction quality evaluation

3.1.1. Combined homogenization/extraction approaches

Out of the *Arabidopsis* metabolites, we selected a group of 16 metabolites, representative for different chemical compound classes, for the pre-LC–MS optimization [16]. Extraction quality parameters absolute recovery (AR) and process efficiency (PE) were then established for the various homogenization and/or extraction procedures, albeit on the more rigid plant tissue material (leaf) only. Process efficiency refers to the combination of (LC–MS) matrix effect and extraction recovery of the analyte from the biological matrix by the sample extraction process [19]. Because the analysis is a multicompound metabolomics analysis, optimal extraction quality parameters are always a compromise, e.g. for AR, it is better to have moderate ARs for many metabolites than to have 100% AR for some and almost nothing for other metabolites. Within each individual sample preparation procedure, we analysed six extracts with metabolites spiked before extraction (PRE), six extracts with metabolites spiked after extraction (POST), six blank extracts (BLANC) and six pure metabolite mixtures (in solvent, PURE). As such, AR was calculated as % [(peak area PRE)/(peak area POST)], PE as [((peak area PRE) − (peak area BLANC))/(peak area PURE)]. As these quality parameters vary per compound and procedure, all individual values are transformed to one single value for each procedure using the transformation function and a geometric mean (see Section 2).

Our study initially focussed on the three most common homogenization approaches in metabolomics analysis: the ball mill (procedure A) [3,8,9], mortar and pestle (procedure B) [3] and Ultra Turrax (procedure C) [7]. Homogenization was performed in 1 ml of 20/60/20 H_2O/MeOH/$CHCl_3$ (v/v/v), thus incorporating in-line extraction. Table 1 shows the value of AR and PE for all the investigated metabolites for extraction procedures A, B, and C, followed by the resulting *V* value after transformation. As can be seen, extraction

Table 1
Absolute recovery (AR), process efficiency (PE) and corresponding *V* value for extraction procedures A, B, and C for the different spiked test compounds, ionized in either positive or negative mode

Compound	AR^A	V^A_{AR}	PE^A	V^A_{PE}	AR^B	V^B_{AR}	PE^B	V^B_{PE}	AR^C	V^C_{AR}	PE^C	V^C_{PE}
ES^+												
Leucine	89.2%	0.9877	91.4%	0.9906	71.4%	0.9528	109.7%	0.9475	131.6%	0.7749	123.3%	0.8532
Cytidine	149.7%	0.5068	110.0%	0.9456	59.4%	0.8798	171.9%	0.0100	105.2%	0.9730	247.3%	0.0100
Phenylalanine	42.8%	0.3705	83.0%	0.9786	63.6%	0.9171	108.8%	0.9528	74.3%	0.9610	91.6%	0.9908
S-Adenosylmethionine	54.7%	0.7839	62.1%	0.9063	24.9%	0.1605	10.2%	0.0559	71.1%	0.9518	30.5%	0.2136
Lactose	74.2%	0.9607	2.9%	0.0146	98.1%	0.9980	−15.6%	0.0100	134.0%	0.7487	1.9%	0.0095
Adenine	100.6%	0.9968	77.8%	0.9691	62.0%	0.9055	51.6%	0.6340	75.6%	0.9643	64.9%	0.9254
Zeatin	76.0%	0.9651	129.1%	0.8006	58.8%	0.8722	57.7%	0.8556	70.0%	0.9480	74.1%	0.9605
Gibberellic acid	84.9%	0.9817	259.6%	0.0100	39.6%	0.3241	88.1%	0.9862	68.8%	0.9438	167.3%	0.0713
Jasmonic acid	88.5%	0.9868	201.0%	0.0100	71.0%	0.9515	58.7%	0.8706	72.3%	0.9555	48.4%	0.4693
Abscisic acid	87.6%	0.9856	230.8%	0.0100	54.7%	0.7838	86.9%	0.9845	106.3%	0.9670	197.7%	0.0100
Epibrassinolide	103.9%	0.9801	177.0%	0.0100	55.1%	0.7987	54.6%	0.7825	80.1%	0.9736	82.8%	0.9784
Chorismic acid	76.4%	0.9661	46.5%	0.4335	44.3%	0.3949	14.6%	0.0830	47.9%	0.4584	17.8%	0.1054
Coumaric acid	92.4%	0.9918	284.8%	0.0100	56.5%	0.8317	85.0%	0.9819	74.1%	0.9606	86.1%	0.9835
Spermidine	32.9%	0.2396	59.7%	0.8829	28.0%	0.1884	44.1%	0.3923	64.2%	0.9212	44.0%	0.3908
ES^-												
ADP-glucose	43.3%	0.3801	17.0%	0.0996	15.0%	0.0859	13.4%	0.0755	54.6%	0.5977	31.2%	0.2202
UMP	49.3%	0.4857	28.9%	0.1977	22.5%	0.1406	18.9%	0.1133	67.5%	0.9349	32.0%	0.2294
	R^A_{AR}	0.7210	R^A_{PE}	0.1305	R^B_{AR}	0.4974	R^B_{PE}	0.2621	R^C_{AR}	0.8608	R^C_{PE}	0.2056

R denotes the geometric mean of the respective *V* values.

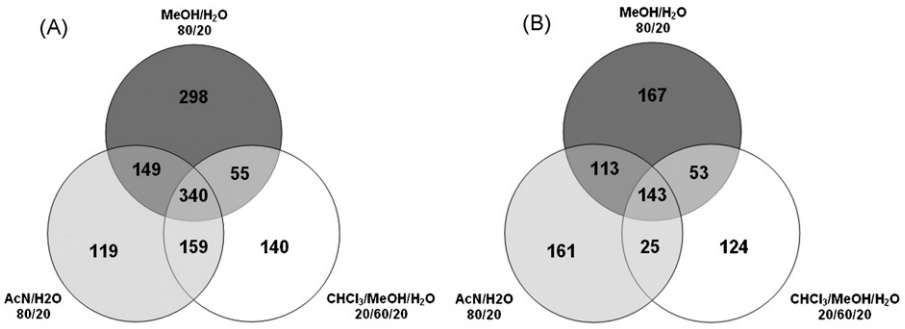

Fig. 2. Venn diagram representing the number of overlapping *m*/*z*-tR signals detected in LC–MS analysis of plant leaves extracts based on different extraction solvent compositions (AcN/H_2O 80/20 (v/v), MeOH/H_2O 80/20 (v/v) and $CHCl_3$/MeOH/H_2O 20/60/20 (v/v/v)) in (A) positive ESI mode and (B) negative ESI mode.

procedure C, with a value R^C_{AR} of 0.8608, shows the most suitable overall absolute recovery (R^A_{AR} 0.7210; R^B_{AR} 0.4974), while procedure B has the best process efficiency with a value R^B_{PE} of 0.2621 (R^A_{PE} 0.1305; R^C_{PE} 0.2056).

Another important quality parameter of extraction in metabolomic research is the number of metabolites extracted. Next to the applied extraction solvent, the disclosure of the, in our case, raw plant material by the homogenization approach will also be of prime importance for the recovery of metabolites. Taking into consideration that all our initial extraction approaches are executed with the same extraction solvent, a mixture of 20/60/20 H_2O/MeOH/$CHCl_3$, and an equal volume of extraction solvent in extraction A, B, and C, a comparison can be made between the different mechanical treatments of the tissues. If we compare the number of *m*/*z*-retention time pairs, e.g. in leaf extracts, a clear contrast is noticeable. Treatment C yields the highest number of detectable *Arabidopsis* *m*/*z*-retention time pairs (thus metabolites) in both ionization modes (375 for positive ESI mode; 93 for negative ESI mode; discarding solvent impurity peaks; $n = 3$), compared with 234 and 147 for treatment A and B in positive ESI mode, 87 and 52 in negative ESI mode, respectively. This favours the Ultra Turrax mixer approach, as it not only excels in freeing up the metabolites from the matrix, noticeable through the higher number of detectable *m*/*z*-retention time pairs, but also provides the highest absolute recovery (see before, Table 1). In terms of repeatability, a key factor in metabolomics, approach C also scores best. On evaluating the relative standard deviation ($n = 6$) of all spiked metabolite peak areas, procedure C (24.25% ± 11.75 for PRE; mean R.S.D.% ± S.D.) also best approximates the results obtained from the pure metabolite mix (21.95% ± 5.44), as compared with the other procedures (39.90% ± 15.65 for procedure A; 45.35% ± 19.81 for procedure B).

Despite its obvious qualities, the in-line homogenization/extraction approach figure of merit in terms of the absolute number of detectable *m*/*z*-retention time pairs, thus metabolites recovered, was still considered less than desired. As concentrating the plant extract by evaporation to dryness/freeze-drying brings along extra sample variation (results not shown), the application of a smaller extraction volume for the plant tissue extraction was further investigated. Some approaches became highly unpractical with extraction volumes below 500 μl. Even procedure C, homogenization with the Ultra Turrax device in eppendorf tubes, becomes impossible with these small volumes. As a consequence, two alternative pre-treatment methods were pursued in which homogenization (with mortar and pestle) was split away from a subsequent extraction step with 200 μl of extraction solvent (off-line). Both the sonication bar (SB; procedure D) as the thermomixer (TM; procedure E) were examined for further processing. Checking the number of detectable *m*/*z*-retention time pairs after extraction clearly shows the benefit of working with small extraction volumes. A number of 737 *Arabidopsis* *m*/*z*-retention time pairs (solvent impurity peaks discarded; $n = 3$) for SB and 763 for TM in positive ionization mode and 300, respectively, 350 in negative ionization mode, were unravelled using the same 20/60/20 H_2O/MeOH/$CHCl_3$ solvent mixture. Although extraction of plant leaves with a sonication bar seems a valuable method, it became clear while exploring the approach that the risks of forming metabolite artefacts through heating of the samples, is substantial. Considering all of this, the mortar and pestle/thermomixer combination using 200 μl of extraction solvent was retained, and put into further in-depth investigation.

3.1.2. Extraction solvent evaluation

The choice of solvent in cold extraction methods has a major impact on the detectable *m*/*z*-retention time pairs, and thus the scope and range of an untargeted metabolite profiling experiment [10]. To expand the range of soluble metabolites, many research groups used a combination of solvents. Within the scope of our reversed-phase LC metabolomics set-up, we considered three solvent compositions as potentially useful: AcN/H_2O 80/20 (v/v) [10], MeOH/H_2O 80/20 (v/v) [10,14,20] and $CHCl_3$/MeOH/H_2O 20/60/20 (v/v/v) [8,9]. Starting from a new pool of homogenized plant leaves, these solvent mixtures have been tested in sample treatment approach E. Our comparison initially focussed on the absolute number of paired *m*/*z*-retention time signals originating from *A. thaliana* (Fig. 2). For the respective solvent mixtures this resulted in 767, 842, and 694 *m*/*z*-retention time pairs for positive ESI mode and 442, 476 and 345 for negative ESI mode ($n = 3$; solvent impurity peaks discarded). As such, this experiment obviously points towards MeOH/H_2O 80/20 (v/v) as the optimum solvent mixture within our LC–MS set-up. As seen in Fig. 2, only 340 individual *m*/*z*-retention time pairs for positive ESI mode and 143 for negative ESI mode were always detected, irrespective of the solvent mixture used, indicating that the range of extracted metabolites is really dependent on the solvent used.

3.1.3. Extraction quality evaluation

Procedure E was finally subjected to a similar, extended extraction quality evaluation as described for the previous procedures. Table 2 outlines the results obtained. A value of 0.8802 and 0.1758 for respectively R^E_{AR} and R^E_{PE} indicates that this approach more than matches up with the previously investigated procedures in terms of extraction recovery. Only for the compounds detected in the negative ionization mode, the results are somewhat less favourable, in comparison. Unsupervised principal component analysis (PCA) of blank extracts (BLANC; B) and extracts with metabolites spiked

Table 2
Absolute recovery (AR), process efficiency (PE) and corresponding *V* value for extraction procedure E for compounds ionizable in positive and negative mode

Compound	AR^E	V^E_{AR}	PE^E	V^E_{PE}
ES^+				
Leucine	86.3%	0.9838	129.6%	0.7962
Cytidine	69.0%	0.9445	35.1%	0.2652
Phenylalanine	73.2%	0.9580	294.7%	0.0100
S-Adenosylmethionine	64.1%	0.9205	5.7%	0.0299
Lactose	89.8%	0.9885	−60.3%	0.0100
Adenine	71.5%	0.9532	51.2%	0.6036
Zeatin	90.1%	0.9889	74.7%	0.9621
Gibberellic acid	67.6%	0.9388	50.6%	0.5539
Jasmonic acid	80.0%	0.9735	68.9%	0.9440
Abscisic acid	86.4%	0.9839	86.5%	0.9841
Epibrassinolide	67.2%	0.9369	21.0%	0.1285
Chorismic acid	68.3%	0.9416	61.2%	0.8984
Coumaric acid	83.6%	0.9796	64.2%	0.9215
Spermidine	58.5%	0.8678	24.1%	0.1539
ES^-				
ADP-glucose	54.0%	0.7626	2.7%	0.0138
UMP	40.1%	0.3303	3.8%	0.0196
	R^E_{AR}	0.8802	R^E_{PE}	0.1758

The resulting geometric mean is addressed to as *R*.

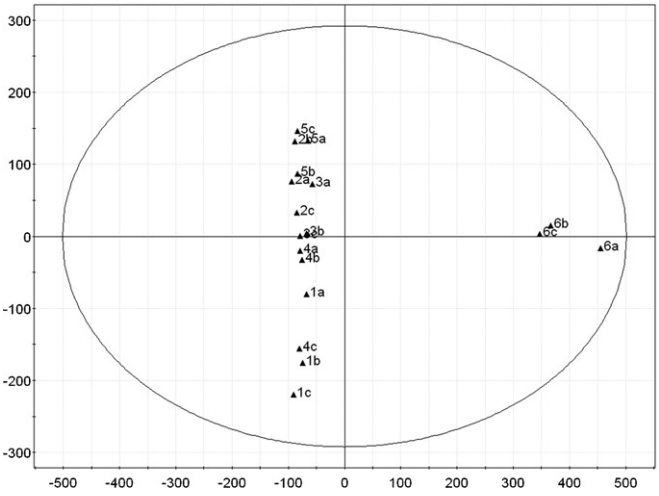

Fig. 4. PCA score plot for the 6 *A. thaliana* leaves extracts (triplicate analysis); extract 6 was defrosted for 1 min during weighing, after which it was treated within the same homogenization and extraction protocol as the other samples.

before extraction (PRE; P) clearly shows the potential of the LC–MS metabolomics set-up, using procedure E, in detecting differential metabolite compositions between biological matrices (Fig. 3). The loading plot identifies *m*/*z*-tR signals originating from the spiked metabolites in PRE as being responsible for the classification of the two plant leaves extracts groups.

On evaluating the repeatability of the procedure, it shows that the R.S.D.% of the spiked metabolite peak areas, 6.66% ± 5.13 (mean R.S.D.% ± S.D.; $n = 6$), is much smaller compared with the previous extraction procedures. A resulting higher extract concentration produces more prominent peaks which in itself promotes a better reproducibility.

The significant effect of small variations in the treatment of samples on the metabolomics outcome was evidenced in the following experiment. We analysed 6 extracts from 6 *A. thaliana* growth plates grown under identical conditions. To add a small variation in the pre-LC–MS treatment of the samples, one sample (extract 6) was defrosted for 1 min during weighing, after which it was treated within the same homogenization and extraction protocol as the other samples. All samples were analysed in triplicate using reversed-phase LC ESI-QTOF-MS in the positive ionization mode. MS data were processed with MarkerLynx®, a part of the MassLynx software package. Subsequently, data were imported in Simca-P® and processed with PCA analysis (Fig. 4). As can be seen in the score plot of the two first principal components, extract 6 shows separate clustering compared to the normally treated samples, clearly proving the importance of a strictly homogeneous pre-analytical process in metabolomics analysis.

If process efficiency data for the most polar compounds, i.e. spermidine, ADP-glucose and UMP, is compared with the average absolute recovery results for these metabolites, there is reasonable evidence for ionization suppression in the polar bulk of the chromatogram. To address this issue more in depth, matrix effect was investigated for this extraction procedure.

3.1.4. Matrix effect assessment

Matrix effect (ME) is an extra but vital parameter in the optimization of an LC–MS method in general and a sample pretreatment procedure in particular. ME refers to alterations of ionization efficiency of metabolites by the presence of coeluting substances and is quantitatively calculated as [1 − (peak area

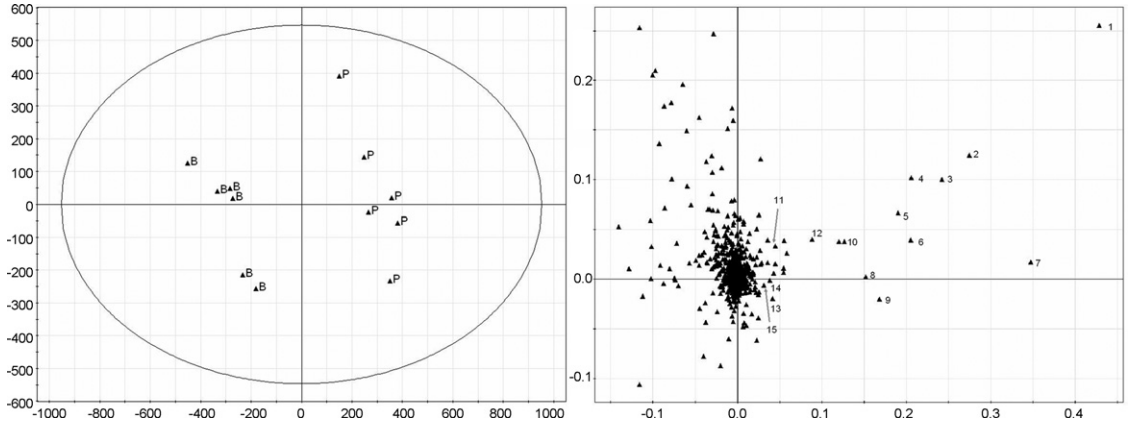

Fig. 3. PCA score plot and loading plot of the LC–MS analysis in positive ionization mode of blank *Arabidopsis* leaves extracts (B) and extracts fortified by metabolite standards (P). The loading plot shows the *m*/*z*-tR signals originating from the spiked metabolite standards (numbered) as responsible for the clear grouping of the samples: (1) abscisic acid $[M-H_2O+H]^+$; (2) abscisic acid $[M-2H_2O+H]^+$; (3) L-phenylalanine $[M+H]^+$; (4) L-phenylalanine $[M-HCOOH+H]^+$; (5) abscisic acid $[M+Na]^+$; (6) coumaric acid $[M-H_2O+H]^+$; (7) zeatin $[M+H]^+$; (8) zeatin $[M-H_2O+H]^+$; (9) adenine $[M+H]^+$; (10) SAM $[M-C_4H_8O_2N+H]^+$; (11) chorismic acid $[M-2H_2O+H]^+$; (12) GA3 $[M-2H_2O+H]^+$; (13) epibrassinolide $[M+H]^+$; (14) cytidine $[M+H]^+$; (15) glutamate $[M+H]^+$.

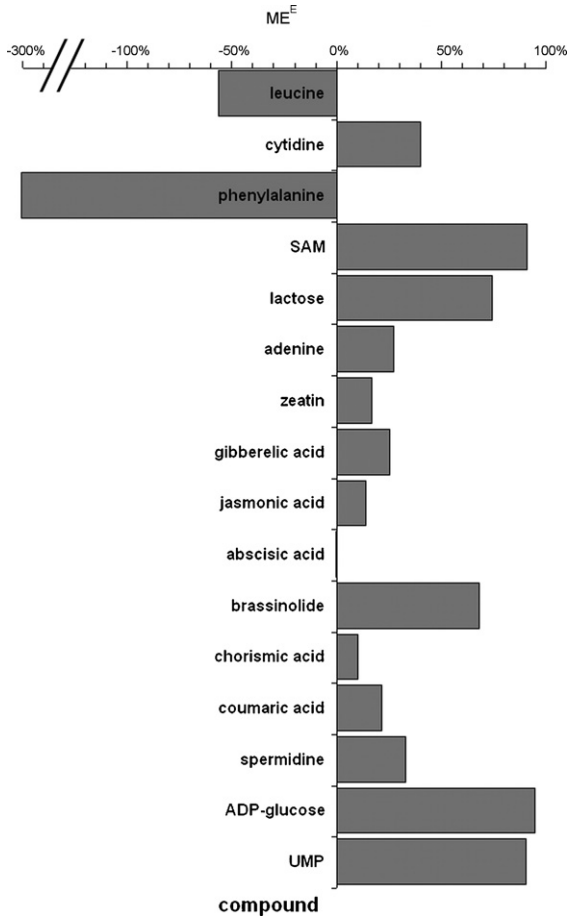

Fig. 5. Evaluation of absolute matrix effects of extraction procedure E through post-extraction addition. Phenylalanine shows a severe ionization enhancement with a value of −309.2%.

POST − peak area BLANC)/peak area PURE] [19]. Fig. 5 shows the matrix effect for all spiked metabolites using procedure E as optimal pre-LC–MS treatment of the samples. Nine of the 16 metabolites show matrix suppression between 0 and 40%. *S*-Adenosylmethionine, lactose, ADP-glucose and uridine-monophosphate show substantial matrix suppression with values above 70%. This confirms our assumption of significant ionization suppression phenomena in the polar bulk of the chromatogram. The mean R.S.D.% of the peak area of these polar metabolites, 6.4% ± 1.6 ($n = 5$), proves that this ionization suppression, however, occurs in a reasonably reproducible way. This indicates the possibility of semi-quantitative measurement, important for metabolomics applications, within the polar front of the chromatogram.

3.2. Repeated extractions

Several metabolomic research groups advocate adding supernatant of a second extraction of the same plant material to the first extraction fraction [13,14,20]. Von Roepenack-Lahaye et al. [13] definitely underlined the redundancy of a third extraction. In our set-up, a second extraction (TM_2) with the same extraction solvent provides a limited gain of 7.85% in the total amount of detectable *m*/*z*-signals, compared with the first extraction (TM_1). 92.15% of the metabolite fraction of TM_2 was analogous to the metabolites extracted in TM_1. To our opinion, a second extraction is not really worth the effort. It adds to the process time and introduces more variation in the sample results, for a limited gain in additional *m*/*z*-retention time pairs.

3.3. Experimental variability: cell cultures and plant leaves

To evaluate our final pre-LC–MS set-up, experimental variability was tested on both *A. thaliana* cell cultures and plant leaves in positive ESI mode. A distinction was made between LC–MS-variability on one hand, comprising the variability of the LC–MS-tool through subsequent injections of the same extract, and technical variability on the other hand, including the extra variability of the pre-LC–MS procedure [13]. A set of 25 *m*/*z*-tR combinations was randomly chosen out of the chromatogram, covering the tR-range and the *m*/*z*-range of our LC–MS tool (Fig. 6). As a result, LC–MS-variability was 8.88% ± 5.16 (mean R.S.D.% ± S.D.; $n = 5$) and 7.05% ± 4.45 ($n = 5$) for plant leaves and cell cultures, respectively. Technical variability, including the variability of our optimal pre-LC–MS set-up, was 12.53% ± 11.21 ($n = 5$) for plant leaves and 9.31% ± 6.65 ($n = 5$) for cell cultures. Comparing LC–MS- and technical variability within both plant materials, only a marginal increase of the variability is visible, indicating a highly reproducible pre-LC–MS procedure.

The number of detectable ions was lower in the cell culture samples. 492 *m*/*z*-tR signals were detected versus 821 in the plant leaves extracts ($n = 3$). Both plant material extracts were also investigated on the number of common *m*/*z*-tR signals, resulting in a number of 93 common signals. This clearly shows the different composi-

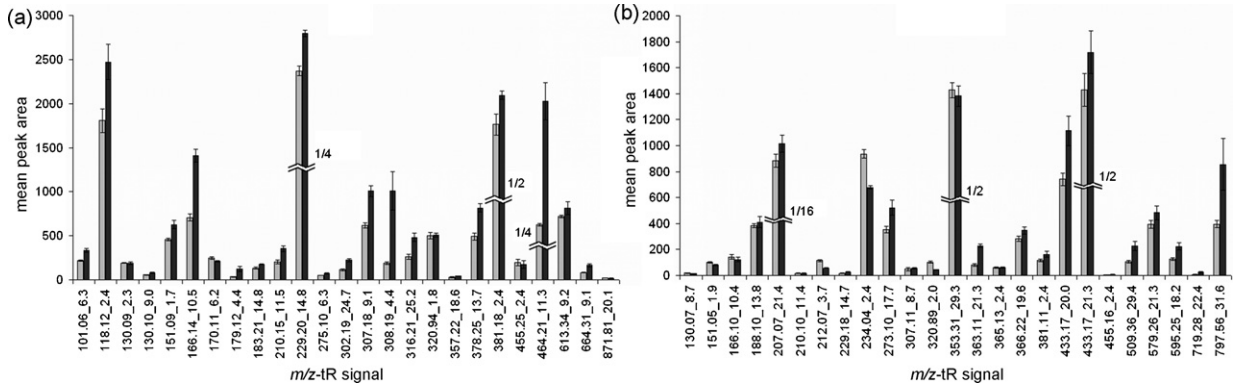

Fig. 6. LC–MS (light bars) and technical variability (dark bars) in LC–MS analysis of cell cultures (a) and plant leaves (b) for 25 randomly chosen *m*/*z*-tR signals covering the tR-range and the *m*/*z*-range of the LC–MS tool ($n = 5$). Some mean peak areas are diminished through division of the peak area (division factors are shown).

tion in metabolite pattern between plant leaves and cell cultures, as already reported by Fukusaki et al. [21] for primary metabolite composition.

4. Conclusion

Pursuing a highly comprehensive and representative image of a metabolome while controlling the experimental variability is of prime importance in metabolomics to compare and quantify different groups of biological samples. The pre-LC–MS methodology plays an important role in this effort. As no gold standard homogenization/extraction set-up has yet been put forward for an LC–MS based plant metabolomics study, we evaluated different homogenization/extraction procedures for a conventional reversed-phase LC–MS based metabolomics tool in order to obtain as much *m*/*z*-tR signals as possible in a reproducible way. Through a transformation function approach, absolute recovery as well as process efficiency could be compared between alternative pre-LC–MS procedures. In-line homogenization and extraction procedures with a higher solvent volume are obviously less reproducible and give a lower *m*/*z*-tR signal yield than an off-line homogenization and extraction method with a lower solvent volume. Homogenization of plant tissue with a mortar and pestle, followed by extraction with MeOH/H_2O 80/20 in a limited volume of 200 μl proved to be the best way of approaching the combined demands in a metabolomics setting. The undesirable outcome of high extraction recoveries and many *m*/*z*-tR signals with poor sample clean-up, resulting in poor LC–MS behaviour was evaluated on the basis of LC–MS matrix suppression. Absolute matrix influence on the LC–MS signal was observed, though without compromising peak detection and above all, without major impact on measurement reproducibility. The LC–MS-variability and technical variability results, in conjunction with the other quality evaluation parameters obtained, clearly point towards an analytical LC–MS set-up advantageous in the differential comparison of biological samples in a metabolomics perspective.

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References

[1] R.D. Hall, New Phytol. 169 (2006).
[2] K. Saito, R.A. Dixon, L. Willmitzer, Biotechnol. Agric. Forest.: Plant Metabol. 57 (2006) 21.
[3] R. De Vos, S. Moco, A. Lommen, J. Keurentjes, R.J. Bino, R.D. Hall, Nat. Protoc. 2 (2007) 778.
[4] O. Fiehn, Plant Mol. Biol. 48 (2002) 155.
[5] O. Vorst, R. De Vos, A. Lommen, R. Staps, R. Visser, R. Bino, R. Hall, Metabolomics 1 (2005) 169.
[6] W. Dunn, S. Overy, W. Quick, Metabolomics 1 (2005) 137.
[7] H.C.J. Orth, C. Rentel, P.C. Schmidt, J. Pharm. Pharmacol. 51 (1999) 193.
[8] P. Jonsson, J. Gullberg, A. Nordstrom, M. Kusano, M. Kowalczyk, M. Sjostrom, T. Moritz, Anal. Chem. 76 (2004) 1738.
[9] J. Gullberg, P. Jonsson, A. Nordström, M. Sjöström, T. Moritz, Anal. Biochem. 331 (2004) 283.
[10] K. Saito, R.A. Dixon, L. Willmitzer, Biotechnol. Agric. Forest.: Plant Metabol. 57 (2006) 65.
[11] S. Moco, R. Bino, O. Vorst, H. Verhoeven, J. De Groot, T. Van Beek, J. Vervoort, R. De Vos, Plant Physiol. 141 (2006) 1205.
[12] G. Le Gall, S. Metzdorff, J. Pedersen, R. Bennett, I. Colquhoun, Metabolomics 1 (2005) 181.
[13] E. von Roepenack-Lahaye, T. Degenkolb, M. Zerjeski, M. Franz, U. Roth, L. Wessjohann, J. Schmidt, D. Scheel, S. Clemens, Plant Physiol. 134 (2004) 548.
[14] C. Böttcher, E. von Roepenack-Lahaye, E. Willscher, D. Scheel, S. Clemens, Anal. Chem. 79 (2007) 1507.
[15] S.G. Villas-Bôas, S. Mas, M. Akesson, J. Smedsgaard, J. Nielsen, Mass Spectrom. Rev. 24 (2005) 613.
[16] R. t'Kindt, G. Alaerts, Y. Vander Heyden, J. Deforce, J. Van Bocxlaer, J. Sep. Sci. 30 (2007) 2002.
[17] T.N. Decaestecker, E.M. Coopman, C.H. Van Peteghem, J. Van Bocxlaer, J. Chromatogr. B 789 (2003) 19.
[18] T.N. Decaestecker, W.E. Lambert, C.H. Van Peteghem, D. Deforce, J. Van Bocxlaer, J. Chromatogr. A 1056 (2004) 57.
[19] P.J. Taylor, Clin. Chem. 38 (2005) 328.
[20] V.J. Nikiforova, J. Kopka, V. Tolstikov, O. Fiehn, L. Hopkins, M.J. Hawkesford, H. Hesse, R. Hoefgen, Plant Physiol. 138 (2005) 304.
[21] E. Fukusaki, K. Jumtee, T. Bamba, T. Yamaji, A. Kobayashi, Z Naturforsch [C] 61 (2006) 267.