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Quantitative profiling of polar primary metabolites of two chickpea cultivars with contrasting responses to salinity

Daniel Anthony Dias[a,1], Camilla Beate Hill[b,*,1], Nirupama Samanmalie Jayasinghe[a], Judith Atieno[c], Tim Sutton[c,d], Ute Roessner[a,b]

[a] Metabolomics Australia, School of BioSciences, The University of Melbourne, Parkville, Victoria 3010, Australia
[b] School of BioSciences, The University of Melbourne, Parkville, Victoria 3010, Australia
[c] Australian Centre for Plant Functional Genomics, School of Agriculture, Food and Wine, The University of Adelaide, Glen Osmond, South Australia, 5064, Australia
[d] South Australian Research and Development Institute, GPO Box 397 Adelaide, South Australia 5001, Australia

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ABSTRACT

This study reports a GC–*QqQ*–MS method for the quantification of forty-eight primary metabolites from four major classes (sugars, sugar acids, sugar phosphates, and organic acids) which can be applied to a number of biological systems. The method was validated in terms of linearity, reproducibility and recovery, using both calibration standards and real samples. Additionally, twenty-eight biogenic amines and amino acids were quantified using an established LC–*QqQ*–MS method. Both GC–*QqQ*–MS and LC–*QqQ*–MS quantitative methods were applied to plant extracts from flower and pod tissue of two chickpea (*Cicer arietinum* L.) cultivars differing in their ability to tolerate salinity, which were grown under control and salt-treated conditions. Statistical analysis was applied to the data sets using the absolute concentrations of metabolites to investigate the differences in metabolite profiles between the different cultivars, plant tissues, and treatments. The method is a significant improvement of present methodology for quantitative GC–MS metabolite profiling of organic acids and sugars, and provides new insights of chickpea metabolic responses to salinity stress. It is applicable to the analysis of dynamic changes in endogenous concentrations of polar primary metabolites to study metabolic responses to environmental stresses in complex biological tissues.

1. Introduction

Salinity and other environmental stresses directly affect the normal growth, development, and reproduction of a plant, and therefore the primary metabolites involved in these processes. The diversity and fluctuation in biological stresses faced by a plant has led to adaptation through biochemical defense mechanisms including both primary and secondary metabolites to directly manage environmental perturbations. With the development of specialized protocols, targeted analysis of primary metabolites can provide a substantial amount of information to investigate complex changes in metabolism caused by different genotypic and/or environmental perturbations.

Chickpea (*Cicer arietinum* L.) is one of the world's most important pulse crops and ranks third in the world for food legume production [1]. Chickpea plants suffer damage even on moderately saline soils that have little impact on bread wheat, which in turn impacts on potential yields of chickpea in rotation with wheat on areas with sub-soil salinity. The reproductive phase is known to be even more sensitive to NaCl exposure than vegetative growth and germination [2], the early development of flower meristems, the conversion of flowers to pods, and the development of seeds in the pods are particularly susceptible to salinity stress. The number of flowers, pods, and seeds is significantly decreased in salt sensitive cultivars compared to tolerant chickpea lines upon salinity treatment [3], and carbohydrate supply to reproductive structures, such as the developing embryo is believed to be a limitation.

Abbreviations: DAS, days after sowing; BSTFA, *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide; CE, collision energy; EI, electron ionization; ESI, electrospray ionization; GC–*QqQ*–MS, gas chromatography-triple quadrupole-mass spectrometry; ISTD, internal standard; LC–*QqQ*–MS, liquid chromatography–triple quadrupole-mass spectrometry; LOQ, limit of quantification; MRM, multiple reaction monitoring; *m*/*z*, mass-to-charge ratio; Pro, proline; PBQC, pooled biological quality control sample; QC, quality control calibration standard mix; RI, retention time index; R^2, linear correlation coefficient; SRM, selected reaction monitoring.

* Corresponding Author.
E-mail address: camilla.hill@unimelb.edu.au (C.B. Hill).
[1] Equal first authors (D.A.D. and C.B.H.).

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Plants have developed an extraordinary genetic diversity controlling the synthesis and regulation of metabolites that has largely been unexplored in the grain legumes. Data based on transcriptomics and proteomics analyses are insufficient to provide an understanding of all aspects of biological processes in response to abiotic stress, since those are ultimately mediated by metabolites. For example, changes in transcript or protein levels do not always correlate to actual changes of cell metabolites due to post-transcriptional and post-translational modifications that modulate protein activities [4]. However, only a few studies on primary metabolism in legumes are available, and these studies cover the model legumes *Medicago truncatula* [5], both model and cultivated legume species of the *Lotus genus* [6,7] and soybean [8].

Metabolomics is now being explored as a possible solution to these problems because it can capture the "ultimate phenotype" of such gene networks and their complex interaction with the environment [9]. Primary metabolites are mostly hydrophilic molecules directly involved in all biochemical processes, including growth, development, and reproduction. Compounds present in carbohydrate metabolism can be challenging to analyze due to the high diversity of compounds with a diverse array of functionalities (including neutral carbohydrates such as saccharides and polyalcohols, polysaccharides, and sugar acids), the often very similar fragmentation of isomers, and the co-elution of two or more compounds with very similar retention times which give rise to complex chromatograms. More effective tools and methods are required for efficient identification and quantification of compound classes, i.e., sugars and organic acids.

Liquid chromatography (LC) coupled to triple quadrupole (*QqQ*)–MS systems have benefited greatly from the high sensitivity and selectivity of tandem MS in the selected reaction monitoring (SRM) mode. Although the coupling of gas-chromatography (GC) to electron impact ionization (EI) mass spectrometry (MS) is one of most well-known and established techniques in analytical chemistry and one of the most developed instrument platforms for metabolite analysis [9]. GC-based methods have suffered a notable delay in the wide acceptance of the *QqQ* analyzer in comparison to LC–MS/MS. GC–MS has been widely used in metabolomics since it has significant separating power, is reproducible, easy to establish and requires a relatively low capital investment compared to other analytical technologies. GC–MS is an ideal analytical technology for the analysis of volatile compounds however most metabolites are not volatile and therefore need to be chemically derivatized in order to make them amenable for GC–MS.

In recent years, there has been a strong emphasis within the metabolomics community that quantitative data is important for biological studies since they describe accurately the actual concentration of the metabolites of interest. New *QqQ* instrumentation allows for higher selectivity and sensitivity and minimizes chromatographic interferences and is typically operated in multiple reactions monitoring (MRM) mode in which collision energies, dwell times and resolution parameters for each individual target compound is optimized using authentic standards, thus, enhancing sensitivity and selectivity [10]. In a single chromatographic run, the application of MRM can simultaneously monitor a large number of MS–MS transitions.

Metabolomics aims to provide a comprehensive and unbiased analysis of all metabolites with a low molecular weight present in a biological sample [4,9]. Due to the structural diversity of metabolites, there is currently no single methodology that can detect the complete metabolome, which is why several extraction methods and instrument platforms are established to analyze highly complex mixtures. Here we used both GC–MS and LC–MS techniques as they are complementary to each other: to accurately quantify primary metabolites of carbon and nitrogen metabolism, GC–MS-based metabolite analysis of organic acids, sugars, sugar alcohols, and sugar acids was carried out on a GC–*QqQ*–MS (Agilent 7890 GC coupled to 7000 Triple quadrupole MS). We have demonstrated the applicability of the method specifically to the extraction of metabolites of flower and pod tissue of two chickpea cultivars, 'Genesis 836' and 'Rupali', before and after salinity stress. To investigate the effects of salinity on other metabolite classes of primary metabolism, we have combined it with an established LC–MS-based metabolomics method for quantification of amine-containing metabolites carried out on a LC–*QqQ*–MS (Agilent 1290 LC coupled to 6490 triple quadrupole MS) according to the standardized protocol developed by [11].

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and solvents were purchased from Sigma–Aldrich (Australia) and were either of analytical or mass spectrometric grades. Deionized water (18.2 MΩ) was produced using a Synergy UV Millipore System (Millipore) was used throughout.

2.2. Plant growth and harvest

The desi chickpea cultivars used in the experiment were Genesis 836 (salt tolerant) and Rupali (salt sensitive). Genesis 836 is a direct introduction from the International Centre for Research in the Semi-Arid Tropics (ICRISAT, Syria), while Rupali was bred by the Department of Agriculture, Western Australia (DAWA), and the Centre for Legumes in Mediterranean Agriculture (CLIMA), The University of Western Australia.

The experiment was conducted in a glasshouse at the University of Adelaide Plant Accelerator facility (Waite Campus, South Australia). Temperature and humidity were controlled and ranged from 24 ± 2 °C and 40% (day), and 18 ± 2 °C and 90% (night), respectively.

Five seeds of each of the cultivars were sown 2 cm deep in pots (19.46 cm height × 14.94 cm diameter) filled with 2.5 kg of 50% University of California (UC) mixture (1:1 peat:sand) and 50% cocopeat (pH 7.5; electrical conductivity ($EC_{1:5}$ 603 μs/cm)). The soil was inoculated with *Rhizobium* inoculum (Group N) prior to sowing. Prior to salt application, plants in each pot were thinned to two uniform plants. At flowering, 21 and 25 days after sowing (DAS) for Rupali and Genesis 836 respectively, each pot received either 0 or 60 mM NaCl (1.3149 g NaCl pot^{-1}) equivalent to applying 100 ml of 0 mM NaCl (untreated pots) or 225 mM NaCl (treated pots) delivered in two increments through the base of the pots by standing the pots in saucers containing saline solution. Each treatment was replicated four times and randomized in a randomized complete block design (RCBD). The pots were watered every two days and maintained at field capacity, 15% (w/w)-determined gravimetrically to maintain salt concentration in the pots and to also avoid salt leaching out of the pots as a result of over watering.

Flowers and pods were harvested and pooled from two plants in each pot 31 and 48 DAS (for cv. Rupali) or 35 and 52 DAS (for cv. Genesis 836). The samples were immediately frozen in liquid nitrogen, and thereafter stored at −80 °C.

2.3. Plant sample extraction and preparation

A modified method for the preparation of plant extracts was used as described previously by [24]. For each chickpea cultivar, approximately 30 mg of frozen flower and pod tissues was weighed into cryomill tubes (Precellys lysing kit, Bertin Technologies). Subsequently, 400 μL of 100% methanol containing 4% internal standard (from a stock solution containing 0.5 mg mL^{-1} $^{13}C_6$-sorbitol and 0.5 mg mL $^{13}C_5$–^{15}N valine) was added to the samples,

followed by vortex-mixing for 30 s, and homogenization (3 × 45 s at 6400 rpm) and −10 °C using a Cryomill (Precellys 24, Bertin Technologies). The samples were then extracted for 15 min at 70 °C in a thermomixer at 850 rpm, and subsequently centrifuged for 5 min at 4 °C and at 13,000 rpm. The supernatants were transferred into new reaction tubes, and 400 μL of water was added into the cryo-mill tubes containing the previously ground tissue pellet. The samples were vortex-mixed for 30 s, and centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatants were then transferred into the reaction tube containing the original supernatant from the previous centrifugation. After the pooled samples were vortex-mixed for 30 s, 5 and 125 μL aliquots of the supernatants were transferred into glass vial inserts and dried *in vacuo* for GC–*QqQ*–MS analysis. In addition, 10 μL aliquots were transferred into glass vial inserts and used for LC–*QqQ*–MS amino acid analysis.

2.4. Gas chromatography–mass spectrometry

2.4.1. Calibration standard sample preparation

Twenty-eight sugars, sugar phosphates, sugar acids, and sugar alcohols, as well as twenty organic acids were purchased from Sigma–Aldrich (Australia). Ten millimole stock solutions were prepared for each individual standard except for 2-ketogluconic acid for which a 2 mM stock solution was prepared. One-hundred and sixty microliters of each sugar standard was subsequently pooled to reach a final volume of 4.48 mL. A 520 μL aliquot of 50% aqueous mixture of methanol was then added to the pooled sugar standards resulting in a final volume of 5 mL with a final concentration of 320 μM. For organic acids, 160 μL of each standard was subsequently pooled to reach a final volume of 4.16 mL, and a 840 μL aliquot of 50% aqueous mixture of methanol was then added resulting in a final volume of 5 mL with a final concentration of 320 μM.

The stock solutions were serially diluted with 50% aqueous mixture of methanol resulting in the following calibration series: 320, 160, 80, 40, 20, 10, 5, 2.5, 1.25 and 0.625 μM calibration points for xylose, malonate, maleate, succinate, fumarate, pipecolate, malate, salicylate, 2-oxoglutarate, aconitate, ferulic acid, raffinose, erlose, and melezitose, respectively; 160, 80, 40, 20, 10, 5, 2.5, 1.25 and 0.625 μM calibration points for itaconate, erythritol, arabinose, ribose, xylitol, rhamnose, arabitol, fucose, citrate, isocitrate, quinate, fructose, mannose, 2-keto gluconic acid, glucose, syringic acid, mannitol, glucuronate, galactitol, gluconate, inositol, uric acid, caffeic acid, fructose-6-phosphate, sucrose, maltose, trehalose, turanose, β-gentiobiose, and melibiose, respectively; 80, 40, 20, 10, 5, 2.5, 1.25 and 0.625 μM calibration points for galactose; and 160, 80, 40, 20, and 10 μM calibration points for nicotinic acid, shikimate, and glucose-6-phosphate, respectively. Forty microliters of each calibration stock was transferred into glass vial inserts, dried *in vacuo,* and stored at −20 °C before subjecting to GC–*QqQ*–MS analysis.

2.4.2. Sugar and organic acid derivatization

All samples were re-dissolved in 20 μL of 30 mg mL^{-1} methoxyamine hydrochloride in pyridine and derivatized at 37 °C for 120 min with mixing at 500 rpm. The samples were incubated for 30 min with mixing at 500 rpm after addition of both 20 μL *N,O*-bis -(trimethylsilyl)trifluoroacetamide (BSTFA) and 1 μL retention time standard mixture [0.029% (v/v) *n*-dodecane, *n*-pentadecane, *n*-nonadecane, *n*-docosane, *n*-octacosane, *n*-dotriacontane, *n*-hexatriacontane dissolved in pyridine]. Each derivatized sample was allowed to rest for 60 min prior to injection.

2.4.3. GC–MS Instrument conditions

Samples (1 μL) were injected into a GC–*QqQ*–MS system comprising of a Gerstel 2.5.2 Autosampler, a 7890A Agilent gas chromatograph and a 7000 Agilent triple-quadrupole MS (Agilent, Santa Clara, USA) with an electron impact (EI) ion source. The GC was operated in constant pressure mode (20 psi) with helium as the carrier gas and using mannitol as a standard for retention time locking of the method. The MS was adjusted according to the manufacturer's recommendations using tris-(perfluorobutyl)-amine (CF43). A J&W Scientific VF-5MS column (30 m long with 10 m guard column, 0.25 mm inner diameter, 0.25 μm film thickness) was used. The injection temperature was set at 250 °C, the MS transfer line at 280 °C, the ion source adjusted to 250 °C and the quadrupole at 150 °C. Helium was used as the carrier gas at a flow rate of 1 mL min^{-1}. Nitrogen (UHP 5.0) was used as the collision cell gas at a flow rate of 1.5 mL min^{-1}. Helium (UHP 5.0) was used as the quenching gas at a flow rate of 2.25 mL min^{-1}. The following temperature program was used; injection at 70 °C, hold for 1 min, followed by a 7 °C min^{-1} oven temperature, ramp to 325 °C and a final 6 min heating at 325 °C.

2.4.4. Method optimization

Individual sugars, organic acids and internal standards were subsequently analyzed on the GC–*QqQ*–MS to obtain retention times and to identify a corresponding unique, precursor ion. For each precursor ion, two product ion scans were carried out using four collision energies (0, 5, 10 and 20 V) to identify product ions in which two product ions were identified. Subsequently, for the two generated products the collision energies for each major reaction monitoring (MRM) transition was optimized using a series of collision energies (CEs) between 0 and 30 V. Collision energy optimization plots for each compound are presented in Additional file 3.

Once collision energies were optimized for each MRM transition, a product ion was selected as the corresponding target ion (T) and the subsequent MRM transition was deemed as the qualifier ion (Q). In some cases, especially for organic acids, a target ion was only provided due to the lack of observable fragment ions and low molecule weight. Absolute concentrations (μM) of targeted sugar and organic acids were quantified using a MRM target ion based on the linear response of the calibration series as described previously. For PBQC samples, additional normalization steps were required to include the weight of the samples as well as the area response for $^{13}C_6$-sorbitol (extraction internal standard).

2.4.5. Method validation

Calibration standards were analyzed for each metabolite to determine retention times, retention time indices relative to the retention time standard mixture, linear correlation coefficient (R^2), recovery, and reproducibility experiments. Calibration curves created for each analyte were fitted using linear regression. Response ratios were calculated relative to the internal standards $^{13}C_1$-mannitol, and the linearity was determined by calculating the corresponding R^2 value. Method reproducibility and recovery was assessed by analysis of calibration standards and of extractions of chickpea samples analyzed in hexaplicates on separate days. The concentrations of the analytes and the standard error of the mean were calculated at each concentration within the linear range of the assay.

2.5. Liquid chromatography–mass spectrometry

2.5.1. Calibration standard sample preparation

Two stock solutions were also prepared: (i) an amino acid solution containing a standard mix of 28 amino acids and amines (4-hydroxyproline, histidine, asparagine, arginine, serine, glutamine, homoserine, glycine, aspartate, citrulline, glutamate, threonine, alanine, γ-aminobutyric acid, proline, cysteine, ornithine, octopamine, lysine, putrescine, tyrosine, methionine, valine, tyramine, isoleucine, leucine, phenylalanine, and trypto-

Table 1
Optimized GC–*QqQ*–MS parameters in multiple reaction monitoring mode (MRM) for the quantification of primary metabolites. Dwell time: 5 s. *Target (T) or qualifier (Q) transition. CE, collision energy.

Compound name	Precursor ion	Product ion	Transition*	CE (V)
Internal standards				
$^{13}C_1$-mannitol	320	130	T	5
$^{13}C_6$-sorbitol	323	132	T	8
Organic acids				
2-Ketogluconic acid	349	201	T	8
	349	186	Q	4
2-Oxoglutarate	198	167	Q	2
	198	154	T	6
Aconitate	375	211	T	4
	375	285	Q	4
Caffeic acid	219	191	T	12
Citrate	183	138.7	T	4
Ferulic acid	308	219	T	4
	308	293	Q	18
Fumarate	245	217	T	6
	245	170.9	Q	12
Isocitrate	257	200.7	T	4
Itaconate	215	132.8	T	18
	259	130.8	Q	20
Malate	233	189	T	2
Maleate	245	216.7	T	4
	245	132.7	Q	12
Malonate	233	216.8	T	2
	233	142.8	Q	8
Nicotinic acid	180	105.9	T	8
	180	135.9	Q	14
Pipecolate	156	83.9	T	6
	156	127.9	Q	6
Quinate	255	239	T	8
	345	255.1	Q	8
Salicylate	267	209	T	8
	209	91	Q	8
Shikimate	255	239	T	4
	204	189	Q	8
Succinate	172	112.9	T	4
	172	155.9	Q	0
Syringic acid	327	312	T	18
Uric acid	456	441.1	T	4
	456	382.1	Q	4
Sugars				
Arabinose	307	217	T	2
β-Gentibiose	361	243	T	6
Erlose	361	169.1	T	10
Fructose	307	217	T	2
Fucose	321	117	T	2
Galactose	319	157	T	4
Glucose	319	129	T	10
Maltose	361	169	T	10
Mannose	319	129	T	6
Melezitose	361	169	T	10
Melibiose	361	169	T	6
Raffinose	361	169	T	10
Rhamnose	364	160	T	4
Ribose	307	217	T	2
Sucrose	361	169	T	6
Trehalose	361	169	T	8
Turanose	361	169	T	6
Xylose	307	217	T	2
Sugar phosphates				
Fructose-6-phosphate	204	189	T	4
Glucose-6-phosphate	364	160	T	4
Sugar alcohols				
Arabitol	319	129	T	4
Erythritol	307	217	T	2
Galactitol	319	129	T	10
Gluconic Acid	423	333	T	4
Glucuronate	364	160	T	4
Inositol	305	217	T	8
Mannitol	319	157	T	4
Xylitol	319	157	T	2

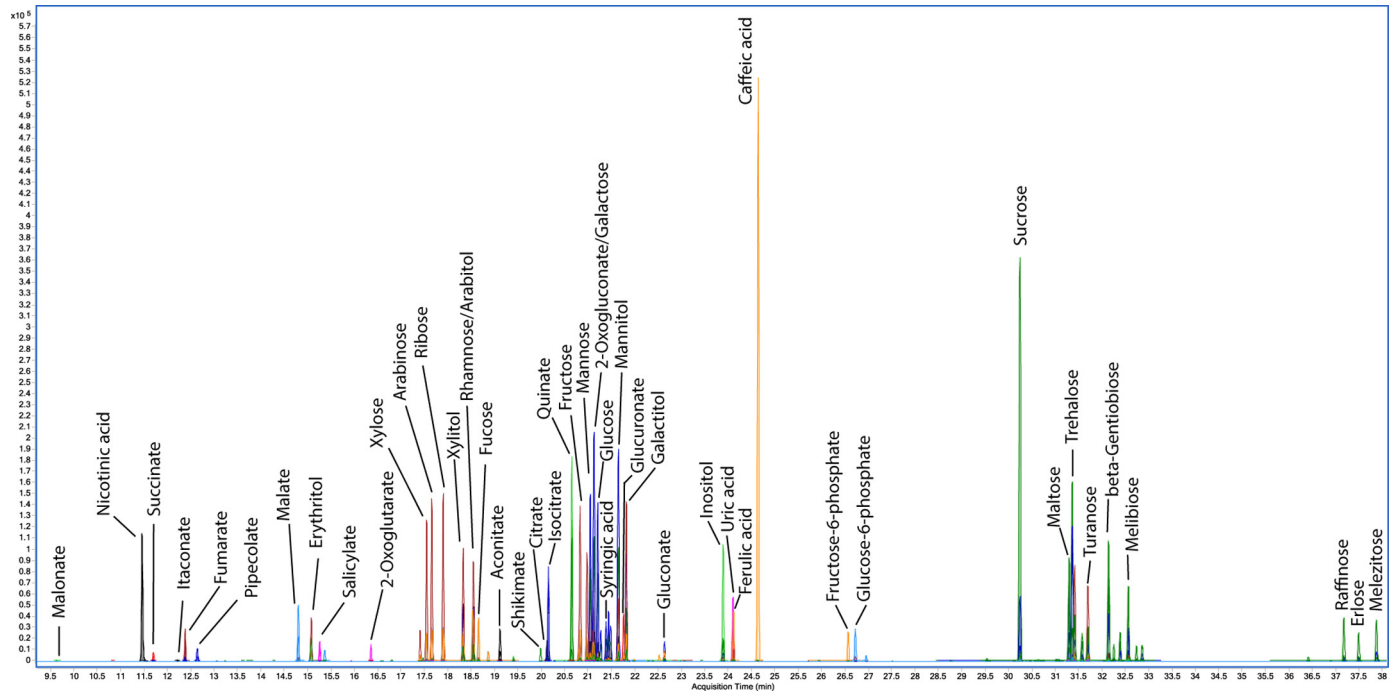

Fig. 1. GC–*QqQ*–MS chromatographic traces showing the separation of 45 primary metabolites within the standard mixes of the developed methodology. Detailed chromatograms are provided (Additional file 2).

phan) in deionized water supplemented with 0.1% formic acid, and (ii) a sulfur-containing compound solution containing glutathione (2.5 mM) and S215 adenosylhomocysteine in deionized water supplemented with 10 mM tris(2-carboxyethyl) phosphine (TCEP) and 1 mM ascorbate. The two stock solutions were mixed and diluted using volumetric glassware with water containing 10 mM TCEP, 1 mM ascorbate and 0.1% formate to produce the following calibration series of combined standards: 0.1, 0.5, 1, 5, 10, 20, 50, 100 and 150 μM.

2.5.2. Amino acid derivatization

For derivatization, an aliquot of each standard or sample (10 μL) was added to 70 μL of borate buffer (200 mM, pH 8.8 at 25 °C) containing 10 mM TCEP, 1 mM ascorbic acid and 50 μM 2-aminobutyrate. The resulting solution was vortexed before adding 20 μL of 6-aminoquinolyl-*N*-hydrosysuccinimidyl carbamate (AQC) reagent [200 mM dissolved in 100% acetonitrile (ACN)] immediately vortexing as described [11]. The 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) reagent was synthesized according to [25]. The samples were heated with shaking at 55 °C for 10 min then centrifuged (13,000 rpm at RT) and transferred to HPLC vials containing inserts (Agilent, springless glass inserts 250 μL) prior to injection.

2.5.3. LC–MS Instrument Conditions

An Agilent 1200 LC-system coupled to an Agilent 6410 Electrospray Ionisation-Triple Quadrupole-MS was used for quantification experiments. The injection volume used for the samples or standards was 1 μL. Ions were monitored in the positive mode using a Dynamic Multiple Reaction Monitoring (DMRM) method optimized for each analyte. The source, collision energies and fragmentor voltages were optimiszed for each analyte by infusing a derivatized standard with LC eluent. The following source conditions were used: sheath gas temperature 315 °C, gas flow 10 L min^{-1}, nebulizer pressure 45 psi and capillary voltage 3800 V. For the chromatography, an Agilent Zorbax Eclipse XDB-C18 Rapid Resolution HT 2.1 × 50 mm, 1.8 μm column was used with a flow rate of 300 μL min^{-1}, maintained at 30 °C, resulting in operating pressures below 400 bar with a 19 min run time as described in Boughton et al. [11]. A gradient LC method was used with mobile phases that comprised of (A) water 0.1% formic acid and (B) acetonitrile 0.1% formic acid (such that at 0.0 and 2.0 min, the % of B was 1 and then increased to 15 and 30% at 9.0 and 14.0 min, respectively, followed by a reduction to 1% at 14.1 and 19.0 min). These conditions provided suitable chromatographic separation of modified amino acids and although co-elution was observed for some of the species, this could be overcome by the mass-selective capabilities of the mass spectrometer using MRM.

2.6. Data processing and statistical analysis

Data were processed using the Agilent MassHunter Workstation Software, Quantitative Analysis, Version B.05.00/Build 5.0.291.0 for quantitation of all compounds. Differences between samples were validated by the Student's *t* -test. Statistical analysis was performed using Excel (Microsoft, www.microsoft.com/).

3. Results and discussion

3.1. Optimization of precursor-to-product ion transitions

The following polar primary metabolites and their internal standards were analysed using GC–*QqQ*–MS: sugars (xylose, arabinose, ribose, rhamnose, fucose, fructose, mannose, galactose, glucose, sucrose, maltose, trehalose, turanose, β-gentiobiose, melibiose, raffinose, erlose, melizitose), sugar phosphates (fructose-6-phosphate, glucose-6-phosphate), sugar alcohols (erithritol, xylitol, arabitol, mannitol, galactitol, inositol), as well as organic acids (malonate, nicotinic acid, maleate, succinate, itaconate, fumarate, pipecolate, malate, salicylate, 2-oxoglutarate, aconitate, shikimate, citrate, isocitrate, quinate, 2-oxogluconate, gluconic acid, glucuronic acid, syringic acid, ferulic acid, uric acid and caffeic acid). $^{13}C_6$-Sorbitol and $^{13}C_5$–^{15}N valine of extraction internal standards which were added into the extraction solvent to compensate for

Table 2
GC–*QqQ*–MS method validation: linearity and recovery. SE, standard error. RI, retention time index. QC, quality control sample. PBQC, pooled biological quality control sample. R^2, linear correlation coefficient.

Metabolite	RI	R^2	QC % recovery(mean + SE)	PBQC % recovery(mean + SE)
Malonate	1046.7	0.9977	105.92 ± 14.68	109.5 ± 14.34
Nicotinic acid	1296.9	0.9977	118.93 ± 12.26	92.24 ± 6.14
Maleate	1300.7	0.9928	91.2 ± 18.28	85.51 ± 5.75
Succinate	1311.7	0.9992	129.23 ± 18.81	106.13 ± 4.79
Itaconate	1339.8	0.9936	103.92 ± 15.6	107.37 ± 5.66
Fumarate	1349.2	0.9982	112.55 ± 13.15	101.87 ± 11.48
Pipecolate	1361.8	0.9982	118.97 ± 21.38	106.5 ± 10.79
Malate	1481.8	0.9972	113.87 ± 12.84	110.64 ± 18.56
Erythritol	1496.8	0.9998	91.24 ± 7.03	103.05 ± 2.09
Salicylate	1506.1	0.9709	125 ± 11.7	82 ± 5.4
2-Oxoglutarate	1577.5	0.9978	114.52 ± 9.24	77.69 ± 12.82
Xylose	1644.1	0.9992	113.9 ± 10.1	97.88 ± 3.39
Arabinose	1660	0.9984	88.93 ± 6.97	100.58 ± 6.35
Ribose	1675.2	0.9985	89.41 ± 7.31	95.86 ± 2.23
Xylitol	1702.5	0.9986	88.78 ± 6.39	114.9 ± 2.23
Rhamnose	1715.2	0.9984	88 ± 7.52	114.63 ± 3
Arabitol	1716.5	0.9985	87.58 ± 6.91	126.66 ± 2.07
Aconitate	1752.1	0.995	120.23 ± 10.4	89.91 ± 10.39
Fucose	1758.4	0.9975	96.51 ± 9.07	89.16 ± 6.53
Shikimate	1807.9	0.9952	121.42 ± 14.98	106.17 ± 4.1
Citrate	1815.6	0.9907	115.62 ± 15.67	106.79 ± 2.52
Isocitrate	1818.1	0.9926	112.57 ± 16.61	109.08 ± 5.99
Quinate	1849.2	0.9984	120.54 ± 16.84	131.25 ± 3.28
Fructose	1860.6	0.9998	86.84 ± 8.96	117.21 ± 17.72
Mannose	1870.1	0.9972	88.21 ± 9.39	106.7 ± 3.03
Galactose	1875.4	0.9986	104.92 ± 7.14	108.71 ± 2.99
2-Ketogluconic acid	1877.8	0.9948	51.31 ± 18.19	97.69 ± 2.18
Glucose	1881.3	0.9948	103 ± 6.43	110.27 ± 24.08
Syringic acid	1896.2	0.9977	85.08 ± 17.05	110.25 ± 5.36
Mannitol	1914.9	0.9957	100.02 ± 7.19	110.53 ± 3.85
Glucuronate	1922.4	0.998	90.35 ± 8.62	100.59 ± 3.21
Galactitol	1926.9	0.9961	101.66 ± 6.69	106.56 ± 1.86
Gluconate	1987.3	0.9985	99.06 ± 13.94	101.99 ± 2.35
Inositol	2080.8	0.9978	84.42 ± 9.68	105.06 ± 3.09
Ferulic acid	2097	0.9951	121.45 ± 16.02	102.71 ± 8.57
Uric acid	2097	0.9931	113.01 ± 18.16	103.3 ± 7.01
Caffeic acid	2136.6	0.9927	100.96 ± 14.22	102.57 ± 7.69
Fructose-6-P	2297.9	0.9963	99.99 ± 7.62	97.95 ± 5.51
Glucose-6-P	2311.4	0.9986	106.79 ± 14.57	104.67 ± 7.1
Sucrose	2627.5	0.9927	48.13 ± 7.5	104.9 ± 6.9
Maltose	2721.9	0.9992	94.3 ± 12.41	93.16 ± 4.05
Trehalose	2727.2	0.9921	79.86 ± 6.56	103.52 ± 4.46
Turanose	2732.6	0.9973	92.15 ± 14.15	84.97 ± 3.87
beta-Gentibiose	2797.9	0.9951	107.07 ± 17.57	99.52 ± 9.77
Melibiose	2842.9	0.9966	90.28 ± 5.8	104.56 ± 10.12
Raffinose	3346.9	0.9844	102.77 ± 6.28	98.14 ± 14.92
Erlose	3385.4	0.9809	117.63 ± 20.38	95.96 ± 24.21
Melezitose	3426.2	0.9931	113.05 ± 14.84	114.78 ± 17.74

the inefficiencies or losses in the extraction and sample preparation steps. $^{13}C_6$-Sorbitol and $^{13}C_5$-^{15}N Valine act as internal standards for GC–*QqQ*–MS analysis and LC–*QqQ*–MS analysis respectively. $^{13}C_1$-Mannitol accounts for the variations during data acquisition in GC–*QqQ*–MS including derivatization, ionization and detection of sugars, sugar acids, sugar phosphates, sugar alcohols and organic acids. Optimal MRM transitions, dwell time, and collision energies were identified using authentic standards for each metabolite (Table 1, Additional file 1).

Fig. 1 and Additional file 2 show the final chromatographic separation achieved for the 45 metabolites using GC–*QqQ*–MS. MS/MS conditions were optimized to produce maximal signal, and plots (intensity vs collision energy) showing the optimization of transitions are provided in Additional file 3.

3.2. Method validation

The validation experiments were conducted to demonstrate robustness, precision, and accuracy of this method, and to ensure that the measured concentrations are close to the unknown content of the metabolite present in real samples. For this study we evaluated the linear correlation coefficient (R^2), higher and lower limits of quantitation, recovery, and reproducibility of the analytical method for quantitation of organic acids, sugar phosphates, sugar alcohols, and sugars, in samples spiked with a known concentration of calibration standard mix as well as samples prepared from pooled biological quality control (PBQC) samples of chickpea tissues (Tables 2 and 3).

3.3. Calibration curve, limit of quantitation, and linearity

Linearity of the calibration curve for each organic acid and sugar was assessed by preparing serial dilutions of the calibration standards mixes ranging from 320 μM to 0.625 μM in triplicate for each metabolite (as detailed in Section 2). The limits of quantification (LOQ) are defined as the highest and lowest analyte concentration, which can be quantified precisely and accurately, and is identical to the lowest and highest point of the calibration curve. A linear regression was used in the calibration curve for all metabolites. For mannitol, galactitol, gluconate, caffeic acid, fructose-6-phosphate,

Table 3
GC–*QqQ*–MS method validation: reproducibility. Values obtained during the validation of the method for quantification of sugars and organic acids in spiked/unspiked QC and PBQC samples. QC, quality control sample. PBQC, pooled biological quality control sample. Mean in μM. SE, standard error, n.d.: not detected, CV: coefficient of variation; concentration of the spike: 50 μM.

Metabolite	QC spiked		QC not spiked		PBQC spiked		PBQC not spiked	
	(Mean ± SE)	CV	(Mean ± SE)	CV	(Mean ± SE)	CV	(Mean ± SE)	CV
2-Ketogluconic acid	83.46 ± 1.68	0.02	64.11 ± ±1.03	0.02	51.4 ± 0.98	0.02	2.56 ± 0.01	0.00
2-Oxoglutarate	118.06 ± 4.56	0.04	61.45 ± 3.37	0.05	72.78 ± 6.92	0.10	33.93 ± 2.09	0.06
Aconitate	127.82 ± 4.97	0.04	67.54 ± 3.03	0.04	52.38 ± 4.42	0.08	7.43 ± 0.51	0.07
Caffeic acid	116.02 ± 4.85	0.04	66.94 ± 1.57	0.02	56.77 ± 3.35	0.06	5.48 ± 0.13	0.02
Citrate	140.62 ± 3.86	0.03	86.33 ± 1.97	0.02	78.39 ± 2.43	0.03	25 ± 1.68	0.07
Ferulic acid	135.33 ± 6.76	0.05	74.16 ± 1.37	0.02	54.84 ± 3.85	0.07	3.49 ± 0.02	0.01
Fumarate	137.24 ± 7.18	0.05	79.35 ± 2	0.03	72.64 ± 4.75	0.07	21.71 ± 1.23	0.06
Isocitrate	133.99 ± 5.08	0.04	80.32 ± 2.22	0.03	60.29 ± 2.64	0.04	5.75 ± 0.2	0.03
Itaconate	134.55 ± 7.71	0.06	81.23 ± 1.62	0.02	56.36 ± 2.6	0.05	2.68 ± 0.31	0.11
Malate	140.7 ± 6.35	0.05	83 ± 1.44	0.02	226.29 ± 3.49	0.02	170.97 ± 10.29	0.06
Maleate	109.58 ± 4.75	0.04	69.65 ± ±3.08	0.04	44.52 ± 2.32	0.05	1.76 ± 0.5	0.28
Malonate	120.78 ± 9.25	0.08	62.98 ± 4.42	0.07	63.56 ± 6.86	0.11	8.81 ± 0.55	0.06
Nicotinic acid	135.55 ± 8.18	0.06	71.66 ± 1.11	0.02	78.35 ± 7.75	0.10	22.3 ± 2.34	0.10
Pipecolate	133.23 ± 9.2	0.07	78.7 ± 2.69	0.03	61.22 ± 4.65	0.08	7.97 ± 0.25	0.03
Quinate	149.72 ± 5.41	0.04	91.79 ± 0.92	0.01	70.12 ± 1.5	0.02	4.49 ± 0.04	0.01
Salicylate	124.31 ± 6.19	0.05	59.27 ± 3.8	0.06	81.63 ± 3.6	0.04	13.5 ± 0.05	0.00
Shikimate	137.77 ± 8.66	0.06	73.26 ± 0.47	0.01	66.45 ± 2.09	0.03	13.36 ± 0.42	0.03
Succinate	139.51 ± 7.39	0.05	76.52 ± 1.38	0.02	138.67 ± 8.65	0.06	86.34±4.57	0.05
Syringic acid	112.93 ± 3.73	0.03	74.45 ± 0.99	0.01	56.18 ± 2.39	0.04	1.06 ± 0.02	0.02
Uric acid	131.75 ± 6.07	0.05	77.26 ± 1.26	0.02	56.34 ± 3.14	0.06	4.69 ± 0.04	0.01
Erlose	114.67 ± 12.15	0.11	47.32 ± 2.68	0.06	49.68 ± 10.84	0.22	1.67 ± 0.04	0.02
Arabinose	124.27 ± 5.37	0.04	75.5 ± 1.21	0.02	56.79 ± 2.7	0.05	6.57 ± 0.21	0.03
Arabitol	137.93 ± 4.85	0.04	90.65 ± 1.14	0.01	64.37 ± 0.91	0.01	1.02 ± 0.04	0.04
β-Gentibiose	116.86 ± 7.48	0.06	60.24 ± 1.41	0.02	58.24 ± 4.72	0.08	8.47 ± 0.41	0.05
Erythritol	125.25 ± 5.81	0.05	74.83 ± 1.27	0.02	52.79 ± 0.93	0.02	1.27 ± 0.01	0.00
Fructose	122.87 ± 5.77	0.05	74.99 ± 0.71	0.01	174.73 ± 6.35	0.04	116.38 ± 1.67	0.01
Fructose-6-P	115.93 ± 4.21	0.04	63.14 ± 1.76	0.03	53.61 ± 2.47	0.05	4.64 ± 0	0.00
Fucose MX1	117.88 ± 6.34	0.05	64.64 ± 0.95	0.01	54.26 ± 3.13	0.06	9.74 ± 0.52	0.05
Galactitol	129.71 ± 4.26	0.03	75.76 ± 0.73	0.01	54.02 ± 0.84	0.02	0.72 ± 0.04	0.04
Galactose	111.02 ± 2.57	0.02	59.18 ± 4.45	0.08	54.46 ± 1.34	0.02	0.1 ± 0	0.00
Gluconate	108.57 ± 7.55	0.07	54.22 ± 1.2	0.02	51 ± 1.05	0.02	n.d.	-
Glucose	126.79 ± 4.29	0.03	72.33 ± 0.96	0.01	227.41 ± 7.93	0.03	172.25 ± 2.94	0.02
Glucose-6-P	113.79 ± 6.95	0.06	56.74 ± 1.22	0.02	54.95 ± 3.82	0.07	6.53 ± 0.01	0.00
Glucuronate	120.37 ± 5.13	0.04	71.66 ± 1.09	0.02	51 ± 1.44	0.03	0.71 ± 0.01	0.00
Inositol	122.87 ± 5.26	0.04	77.2 ± 1.42	0.02	69.62 ± 1.7	0.02	16.67 ± 1.12	0.07
Maltose	112.59 ± 5.18	0.05	63.54 ± 1.81	0.03	49.39 ± 1.81	0.04	2.82 ± 0.01	0.00
Mannitol	125.36 ± 4.52	0.04	71.94 ± 0.82	0.01	55.74 ± 1.72	0.03	0.48 ± 0.02	0.03
Mannose	121.61 ± 4.99	0.04	74.16 ± 0.8	0.01	53.35 ± 1.36	0.03	n.d.	-
Melezitose	121.59 ± 13.14	0.11	52.02 ± 2.25	0.04	63.29 ± 7.99	0.13	5.94 ± 0.08	0.01
Melibiose	117.96 ± 7.17	0.06	62.33 ± 1.56	0.02	57.25 ± 4.57	0.08	4.98 ± 0.07	0.01
Raffinose	133.74 ± 16.41	0.12	60.25 ± 1.04	0.02	69.66 ± 6.75	0.10	20.59 ± 0.07	0.00
Rhamnose	126.78 ± 5.51	0.04	78.46 ± 1.01	0.01	57.52 ± 1.26	0.02	0.52 ± 0	0.00
Ribose	124.76 ± 5.48	0.04	75.76 ± 1.22	0.02	48.54 ± 1.34	0.03	0.61 ± 0.01	0.00
Sucrose	91.39 ± 3.03	0.03	66.06 ± 1.27	0.02	86.25 ± 2.53	0.03	33.73 ± 0.69	0.02
Trehalose	117.41 ± 3.11	0.03	78.39 ± 1.97	0.03	51.76 ± 2	0.04	n.d.	-
Turanose	110.73 ± 6.43	0.06	61.66 ± 1.5	0.02	43.85 ± 1.9	0.04	1.14 ± 0.36	0.32
Xylitol	126.13 ± 4.96	0.04	77.76 ± 0.95	0.01	57.69 ± 1	0.02	0.24 ± 0.01	0.01
Xylose	139.1 ± 7.57	0.05	75.91 ± 0.96	0.01	52.55 ± 1.52	0.03	3.62 ± 0.01	0.00

and β-gentiobiose, a weighting of $(1/x)$ would then be applied to calculate the concentration(s) for metabolites detected at the lower range of the calibration curves. For all other analytes no weighting factor was applied. Thus, these factors and regression were applied in every analytical curve during the whole validation study. Response ratios were calculated relative to the internal standard $^{13}C_1$-mannitol. R^2 values ranged from 0.9809 (for erlose) to 0.9998 (for both erythritol and fructose) (Table 2).

3.4. Recovery

The recovery of an analyte from a biological matrix is based upon the efficiency of the solvent(s) of choice in the extraction process as well as the instrument response which can affect the determination of final concentrations [12–14]. Recovery was calculated by comparing the amount of each metabolite present in (i) a calibration standard mix (QC) with a concentration of 80 μM spiked with additional 50 μM of the same calibration standard mix, as well as in (ii) PBQC sample (100 μM and 5 μM) spiked with the calibration standard mix (80 μM). All recovery tests were prepared in hexaplicates, and the values of recovery for the QC and PBQC samples are provided in Table 2.

The recovery values for the spiked QC samples were high (90–110%) for 28 out of the analyzed 48 metabolites, including fucose (100%), pipecolate (99%), and erythritol (99%). However, for 2-keto gluconic acid and sucrose the overall recoveries were 34% and 49%, respectively. Several sugars, including erlose (135%), raffinose (147%), and melezitose (133%), show a slightly higher recoveries as previously reported [15–18].

The recovery values for the spiked PBQC samples were high (90–110%) for 37 out of the analysed 48 metabolites, including nicotinic acid (101%), arabinose (99%), and glucuronate (100%). Only arabitol (127%) and quinate (126%) showed slightly higher recoveries as previously reported [15–18]. For most metabolites recovery

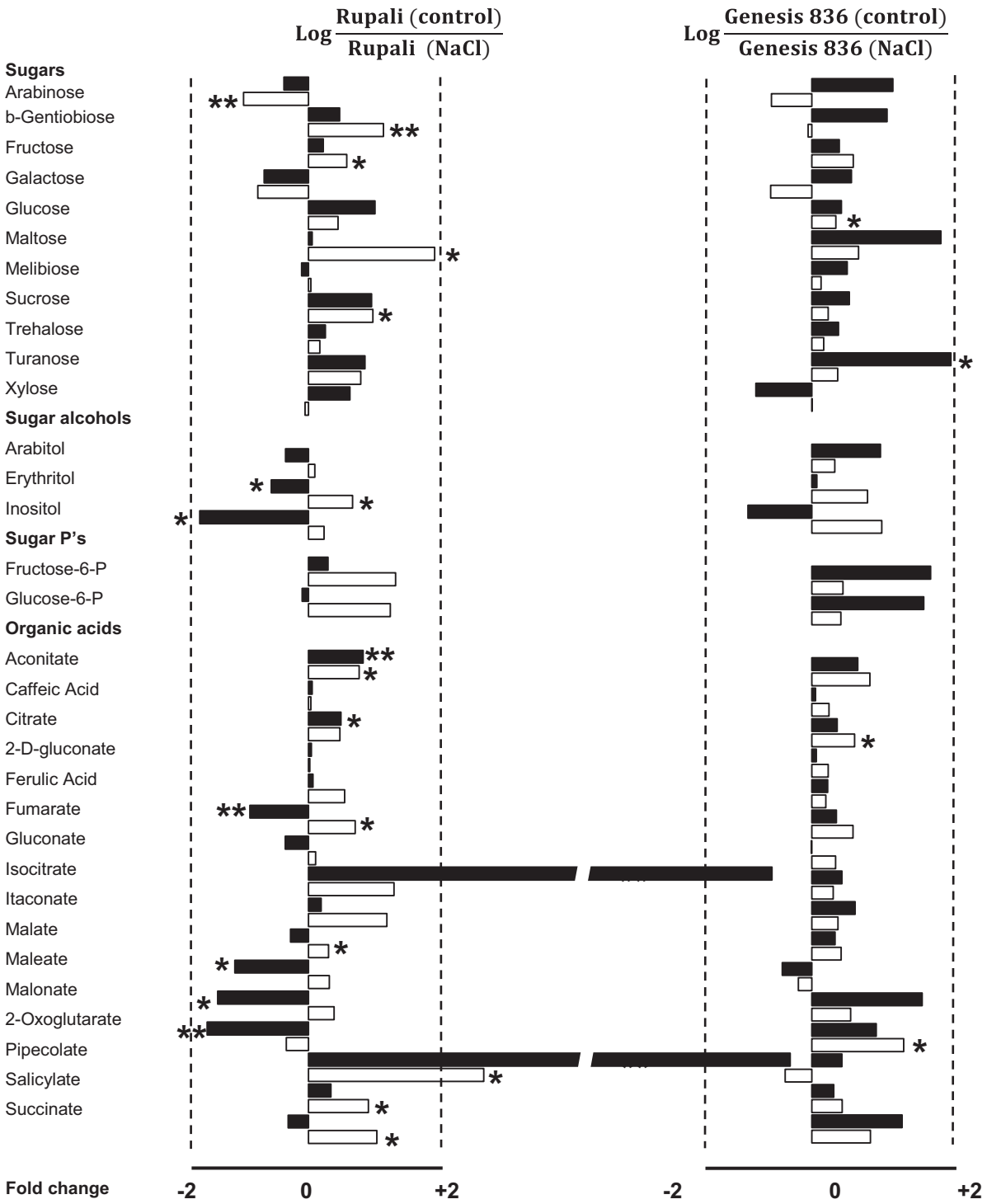

Fig. 2. Logarithmic ratios of sugar and organic acid contents in flowers (black bars) and pods (white bars) of chickpea cvs Rupali (left column), and Genesis 836 (right column). Comparisons are made before (control) and after treatment with 60 mM NaCl for four weeks. Relative response ratios were calculated using the metabolite peak area divided by both the peak area of the internal standard and the sample dry weight (g). Fold changes were calculated by dividing the response ratios of control by the response ratios of salt treated per line. Values that are significantly higher at the $^{*}P < 0.05$ and $^{**}P < 0.01$ level are indicated with asterisks. The threshold of a ±2-fold change is indicated as a dashed line. P: phosphate

values for the spiked versus unspiked PBQC samples were in the range of 72–110% which is in close agreement with previous reports for polar metabolite determination via GC–MS [18].

3.5. Reproducibility

The validation of reproducibility (standard error) assesses the variability observed within an instrument over a short period to assess the method's accuracy and precision. Reproducibility was evaluated by spiking (i) a calibration standard mix (QC) with a concentration of 80 μM with additional 50 μM of the same calibration standard mix, as well as spiking (ii) 100 μM of PBQC sample with the calibration standard mix (80 μM). For malonate, malate, shikimate, citrate, fructose, glucose and sucrose only 5 μL of PBQC standard mix was used. All reproducibility tests were prepared in hexaplicates. The determined variability values (standard errors)

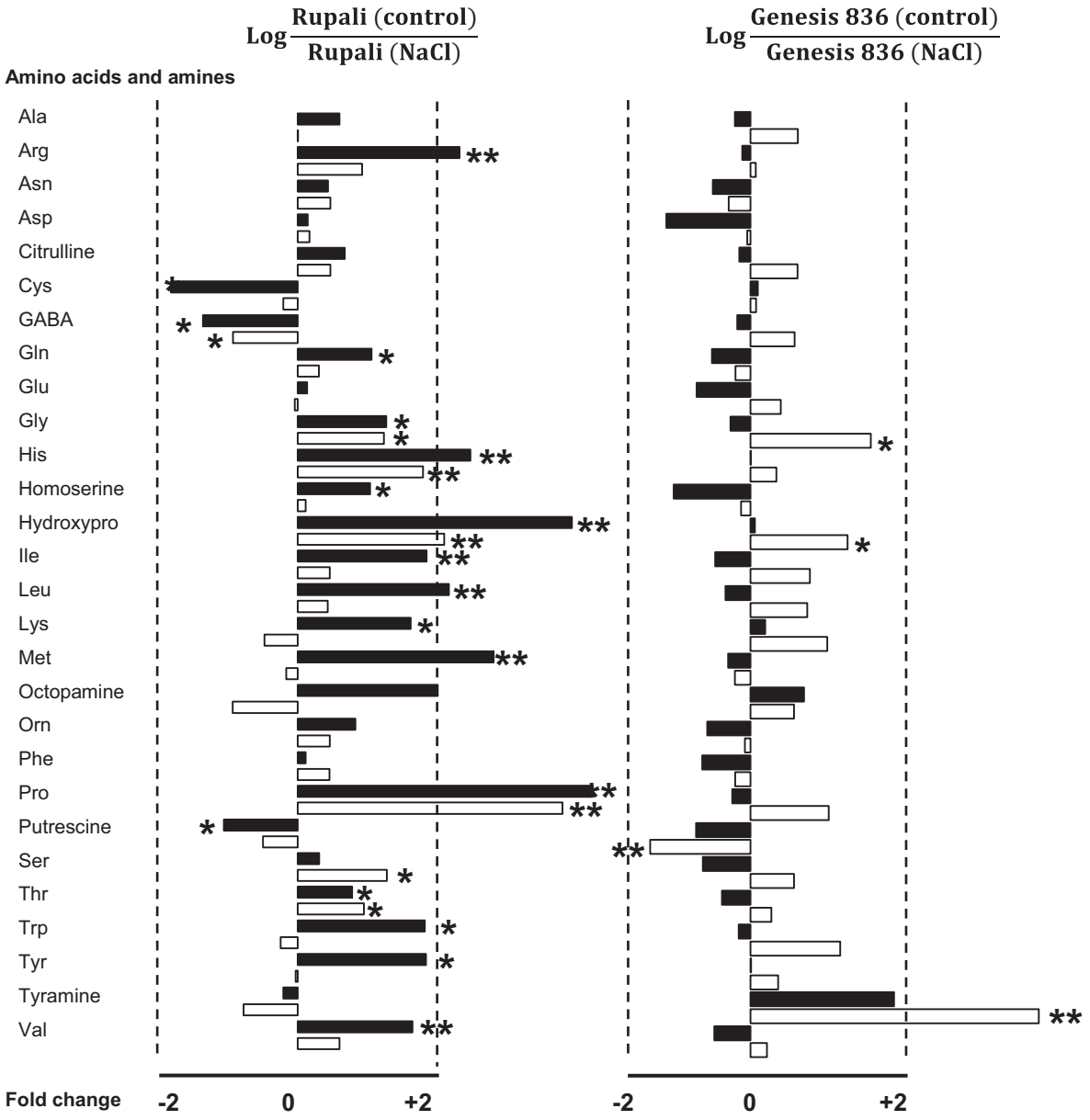

Fig. 3. Logarithmic ratios of amino acid and amine contents in flowers (black bars) and pods (white bars) of cvs Rupali (left column), and Genesis 836 (right column). Comparisons are made before (control) and after treatment with 60 mM NaCl. Other details are the same as in the legend to Fig. 2.

are listed in Table 3. The standard error for practically all metabolites were below 10% for both the spiked calibration standards and PBQC samples, indicating that this method is precise and accurate for quantification of organic acids and sugars. These levels of variability are broadly in agreement with previously reported values for metabolite profiling of different compound classes using GC or LC-MS/MS systems [15–19].

3.6. Comparison of primary metabolite concentrations of tissues from two chickpea cultivars upon salinity stress

Following the validation of the GC–*QqQ*–MS method, it was applied to study metabolic responses in flower and pod tissue of the desi-type chickpea cultivars 'Genesis 836' and 'Rupali' which differ in their tolerance to salinity [20]. Cv. Rupali is salt sensitive, whereas cv. Genesis 836 is more tolerant to salt, and plants of both cultivars were grown in the greenhouse under control and salinity stress (60 mM NaCl for four weeks). To account for differences in maturity, salt treatment was performed 21 days after sowing (DAS) for cv. Rupali, and 25 DAS for cv. Genesis 836. Flowers and seeds were harvested after 31 and 48 DAS (for cv. Rupali) or 35 and 52 DAS (for cv. Genesis 836), and immediately frozen in liquid nitrogen (N_2). Frozen tissue (~30 mg) was extracted in 50 % methanol-water solution including the internal standards $^{13}C_6$ -sorbitol and $^{13}C_5$–^{15}N valine, and organic acid and sugar concentrations were analyzed using GC–*QqQ*–MS using three replicates per cultivar and treatment.

The objective of this part of the study was to understand how salt imposition affects the primary metabolite profile of flowers and pods of the different chickpea cultivars. Therefore, pairwise comparisons between the concentrations of sugars and organic acids of flowers and pods before and after salinity stress for Rupali (Fig. 2, left column) and Genesis 836 (Fig. 2, right column) was performed (Additional file 4). Unless otherwise stated, only metabolite changes which are considered as statistically significant (Student's *t*-test p-value < 0.05) will be discussed.

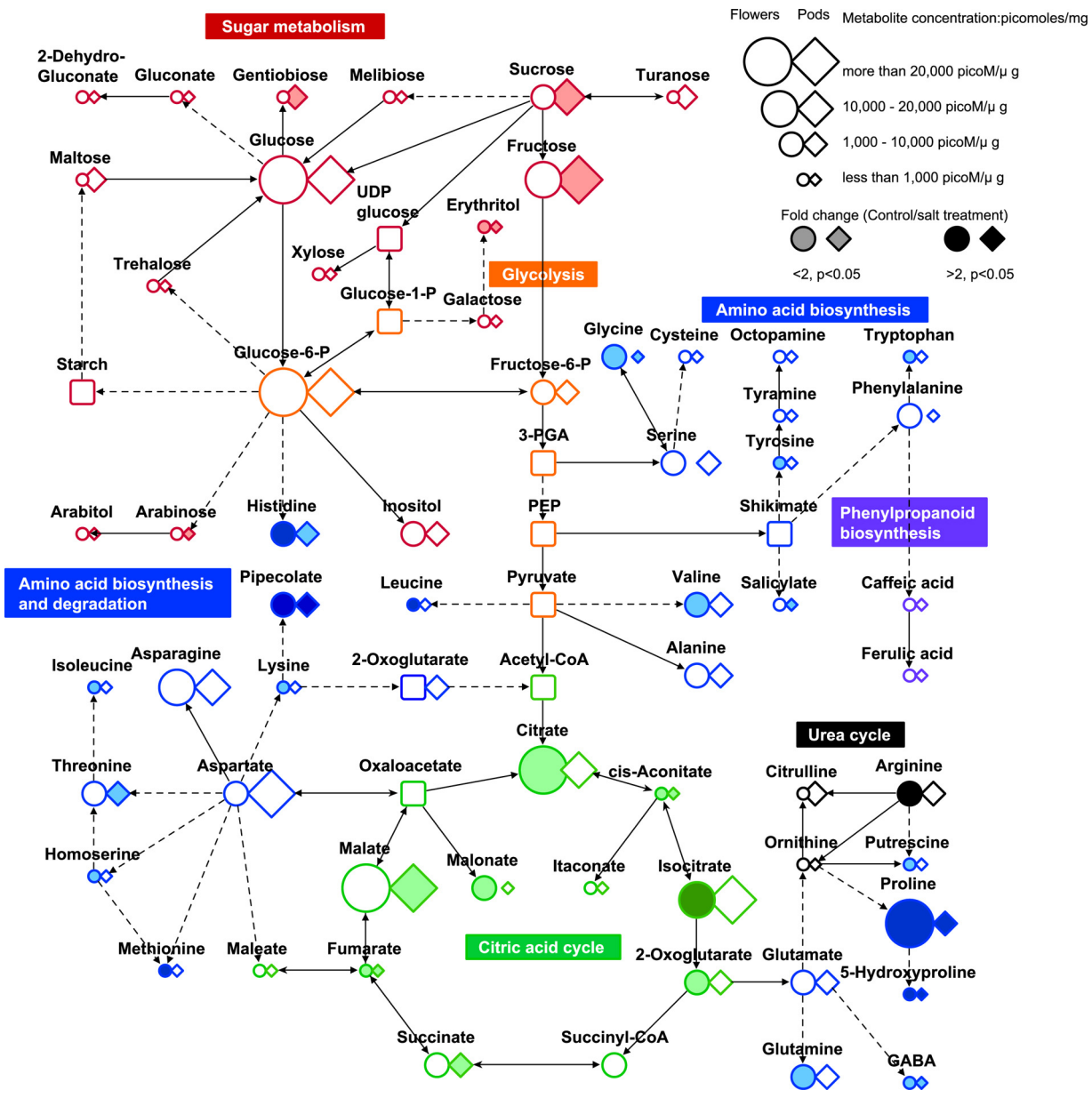

Fig. 4. Metabolic pathway altered by salinity treatment in flowers and pods of chickpea cv Rupali. Changes in concentration are indicated by the size of either the circles (for flowers) or rhombi (for pods). Metabolites that were not measured in this study are depicted by squares. Metabolites that show significant changes ($P > 0.05$) after salinity stress of less than two-fold are highlighted with light colors, whereas significant changes of more than two-fold are highlighted with dark colors. More details are provided in the legend.

In total, eleven sugars, three sugar alcohols, two sugar phosphates, and sixteen organic acids could be detected and quantified in both tissues of the cultivars. Profiles of the metabolites in Rupali show small reductions in the concentration of arabinose (−1.4-fold), but also small increases in the concentrations of β-gentiobiose, fructose and sucrose in pods of between 1.2 and 1.4-fold after salinity stress. Furthermore, erythritol and inositol levels were depleted in flowers, but increased (erythritol) or unchanged (inositol) in pods after salinity stress.

Noticeably, the magnitude of changes was generally larger for organic acids, with particularly pipecolate showing a large increase (flowers and pods, 12.6 and 2.5-fold, respectively) after salinity stress. The tricarboxylic acid (TCA) cycle metabolites isocitrate (flowers, 11.5-fold), cis-aconitate (flowers and pods, 1.33-fold and 1.31-fold, respectively), citrate (flowers and pods, both 1.18-fold), fumarate (pods, 1.28-fold), and malate (pods, 1.11-fold) also showed large salinity stress-induced increases.

Fewer differences in sugar levels were noted in flowers and pods of the salinity tolerant cultivar Genesis 836 compared to Rupali, with only turanose showing a small but significant increase of 1.8 fold after salinity stress. No significant changes were detected for sugar phosphates or sugar acids, and only two small but significant increases in the levels of the organic acids citrate and 2-oxoglutarate in pods were measured after salinity stress.

3.7. *Alteration of amino acid and amine contents after salinity stress in two chickpea cultivars*

To accurately quantify additional metabolites of the carbon and nitrogen metabolism, LC–MS-based metabolite quantification of amino acids and amines was carried out on a LC–*QqQ*–MS accord-

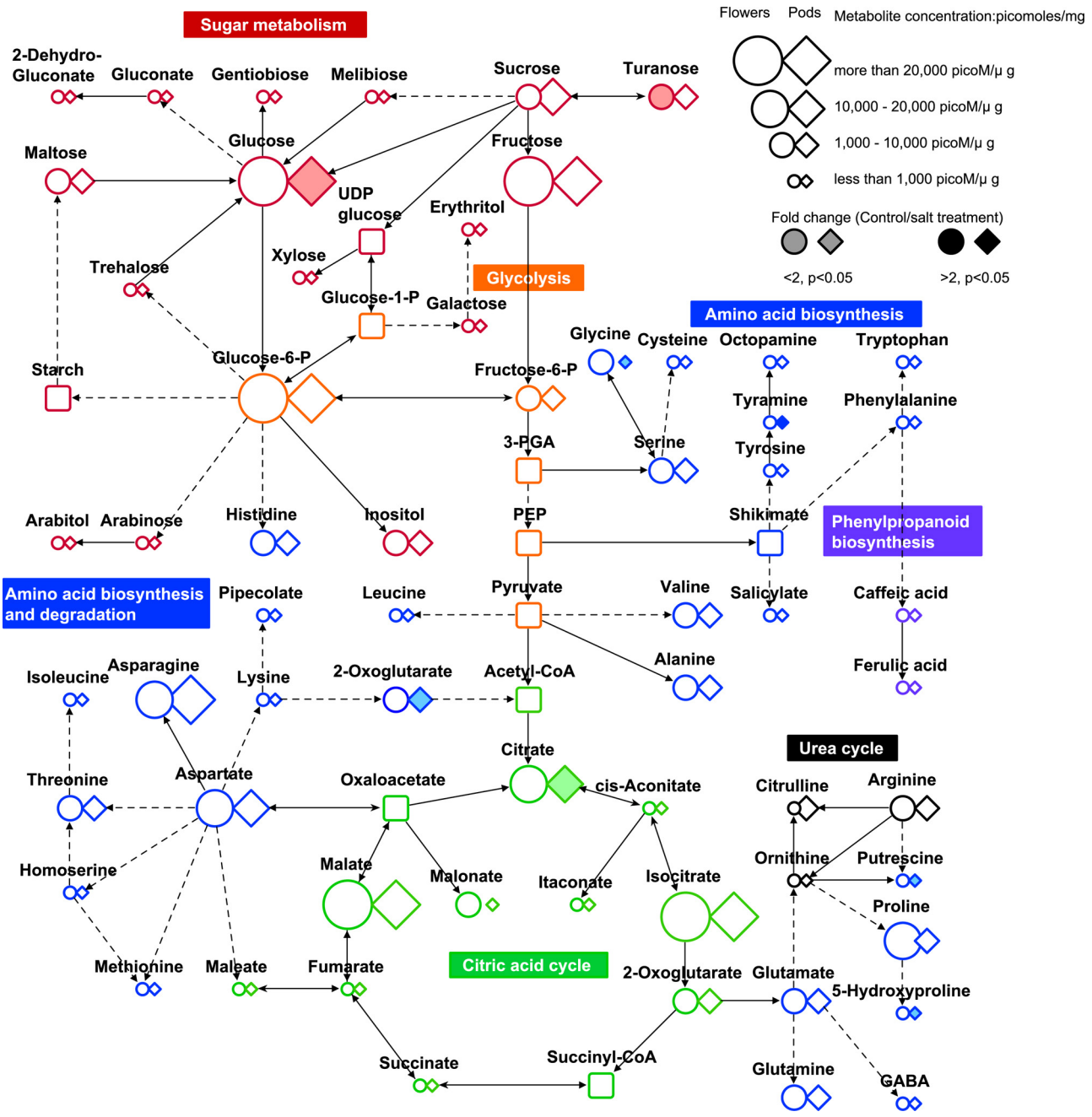

Fig. 5. Metabolic pathway altered by salinity treatment in flowers and pods of chickpea cv Genesis 836. Other details are the same as in the legend to Fig. 4.

ing to the standardized protocol developed by Boughton et al. [11] (Fig. 3, Additional file 4).

Striking differences were seen in the amino acid concentrations in flowers following salt treatment of the salt sensitive cv. Rupali compared to the salt tolerant cv. Genesis 836: In cv. Rupali, 14 out of the 28 measured amines and amino acids (Arg, Glu, Gly, His, homoserine, hydroxyproline, Ile, Leu, Lys, Met, Pro, Thr, Trp, Val) had significantly higher concentrations of between 1.4 and 4.7-fold, and three (Cys, GABA, putrescine) had significantly lower concentrations of between 1.5 and −1.9-fold after salinity treatment compared to control conditions. With the exception of Trp, the concentrations of these amino acids and amines were also much higher in salt affected flowers than pods. On the other hand, cv. Genesis 836 only showed very few significant changes in amino acid concentrations: Gly, hydroxyproline, and tyramine concentrations increased between 1.5 and 3.5-fold, and putrescine decreased −1.5-fold in pods after salinity treatment. Interestingly, the vast majority of amino acids and amines measured in this study were depleted (although not significantly) in flowers of Genesis 836 after salinity stress, whereas the opposite trend is seen in Rupali, where the vast majority of amino acids and amines increased in flowers after salinity stress.

To compile information on connections between all primary metabolites quantified in this study upon salinity exposure and to determine possible sites of metabolic regulation, we created an author-generated metabolite pathway map of primary metabolism of cv Rupali (Fig. 4) and cv Genesis 836 (Fig. 5). Both maps show metabolite concentrations in flowers and pods, the fold change and the significance of changes after salinity stress. Although the overall concentrations of the metabolites are nearly identical after salinity

stress for both cultivars, the primary metabolite pathway map of the salt-sensitive cv Rupali shows significantly higher concentrations for amino acids derived from glucose-6-phosphate, aspartate, and pyruvate upon exposure to salt than in the salt-tolerant cv Genesis 836. The elevated amino acid levels can be a reaction to salt stress and related to tissue damage in the salt-sensitive cultivar rather than a plant response associated with tolerance, which is supported by many other recent studies studying the effect of salinity on the metabolite levels in *Arabidopsis thaliana*, *Lotus japonicus*, *Oryza sativa*, *Hordeum vulgare*, and *Triticum aestivum* [7,21,22]. Although many studies suggest a link between increased levels of osmolytes such as proline and sugars, as well as an induction of metabolic pathways including glycolysis and sugar metabolism, and salinity tolerance, the results in this study do not support this: Both Pro and its hydroxylation product 5-hydroxyproline as well as sucrose and fructose were significantly increased in the salt-sensitive cultivar Rupali but not in salt-tolerant Genesis 836 after salinity stress, supporting recent findings that the contribution of Pro to osmotic pressure being relatively small [23] and not always correlated with enhanced salinity tolerance [22].

However, many organic acids from the TCA cycle including isocitrate and aconitate were also increased in cv Rupali upon stress but not in cv Genesis 836, consistent with recent findings showing that salt-sensitive plant lines use these metabolites increasingly as precursors for *de novo* amino acid biosynthesis after exposure to salt. As these changes in the pool of metabolites from the TCA cycle coincided with a decrease of several sugars (particularly in pods), this points towards an increased rate of glycolysis to provide carbohydrates for use in the TCA cycle, which in turn provides precursors for other reactions such as amino acid synthesis and organic acid as well as chemical energy in the form of adenosine triphosphate (ATP) and reduced nicotinamide adenine dinucleotide (NADH) at an increased rate to support plant survival under salinity stress.

4. Conclusions

Despite improvements in analytical techniques for GC–*QqQ*–MS, the comprehensive analysis and quantitation of metabolites in complex samples remains a challenge, particularly when they are present at varying concentration levels. The ability to detect a large number of polar metabolites in low concentrations in a single analysis offers important benefits compared to other analytical methods. We developed a profiling method which was validated for the quantification of more than 40 metabolites from four major classes of polar compounds, including sugars, sugar alcohols, sugar phosphates, and organic acids. This method was applied to flower and pod samples from two chickpea cultivars differing in their ability to tolerate salt. In this study we were unable to find any evidence that Pro, the most highly studied osmoprotectant, was affected by salinity when comparing metabolite concentrations in the control and the salt-treated samples of the tolerant and the intolerant chickpea line. Instead, we conclude that metabolic differences between cvs Rupali and Genesis 836 following salt stress involve metabolites involved in carbon metabolism and in the TCA cycle, as well as amino acid metabolism. The integration of the developed metabolite profiling method provided unambiguous metabolite identities and absolute quantitative data. Although the method was applied to the analysis of chickpea samples, it is equally applicable for metabolic profiling of other biological samples as the majority of the metabolites play key roles in central biosynthetic pathways.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2015.07.002

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