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Optimization of a gas chromatography–mass spectrometry method with methyl chloroformate derivatization for quantification of amino acids in plant tissue

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ABSTRACT

Rapid, easy and reliable quantification of amino acids is crucial in research on plant amino acid metabolism and nutritional improvement of crops via enrichment of essential amino acids. A recently reported analysis method, based on solid phase extraction (SPE), derivatization with methyl chloroformate and gas chromatography-mass spectrometry was optimized and tested on three-week-old *Arabidopsis thaliana* leaf tissues. Optimization of the SPE cleanup yielded recovery rates of minimum 95% for all amino acids (except arginine). Variations in accuracy and precision did not exceed 12.5%, except for cysteine, histidine and tryptophane, which were excluded from analysis. Quantification of overlapping peaks for isoleucine/threonine and proline/asparagine was possible by selection of two specific fragment ions for each amino acid. Of the 16 selected amino acids, 14 were quantified successfully in at least 75% of the samples, while methionine and tyrosine were only quantifiable in 6% and 42%, respectively. A case study on the aspartate super pathway confirmed the applicability of the optimized method on wild type and genetically modified plants: external supplementation of methionine or lysine yielded a 146-fold or 27-fold increase in the respective absolute amino acid levels compared with the control treatment. Induced expression of *dhdps-r1* (a mutated lysine biosynthesis gene encoding a feedback insensitive enzyme) caused an 83-fold increase in absolute lysine levels.

1. Introduction

Amino acids are important components in all organisms, mainly as building blocks for proteins, but also as neurotransmitters at synapses in the human nervous system [1], precursors for important molecules such as *S*-adenosyl methionine [2], vitamins [3] and nucleotides [4], or as a compound with multiple roles in biotic and abiotic stress responses in plants (e.g., proline) [5]. The metabolism of the essential amino acids in plants is of high importance to improve the nutritional value of certain crops. The aspartate super pathway is a metabolic pathway unique to plants and bacteria, which is responsible for the biosynthesis of four of the nine essential amino acids: lysine (Lys), methionine (Met), threonine (Thr) and isoleucine (Ile), which are all produced from the common precursor aspartate (Asp) (Supplemental Fig. S1). The balance between these amino acids is tightly maintained through a variety of regulatory mechanisms of which feedback inhibition is probably the most important (reviewed in Ref. [6]). In *Arabidopsis thaliana*, for example, Lys controls its own concentration by inhibiting its biosynthesis mainly through feedback inhibition of the two isoforms of the rate-limiting enzyme 4-hydroxy-tetrahydrodipicolinate synthase (HTDPS1 and HTDPS2; formerly known as DHDPS1 and DHDPS2) and by enhancing its own degradation through activation of lysine ketoglutarate reductase/saccharopine dehydrogenase (LKR/SDH) [7].

Since four essential amino acids are produced in this pathway, it has been a frequent target for biotechnological modifications aiming to improve crop nutritional value [8]. An adequate, accurate and easy to use quantification method to analyze multiple amino acids in these genetically modified crops is therefore essential. In previous studies using a high performance liquid

Abbreviations: HTDPS, 4-hydroxy-tetrahydrodipicolinate synthase; LKR/SDH, lysine ketoglutarate reductase/saccharopine dehydrogenase; MGL, methionine-γ-lyase; RII, relative ion intensity; RRT, relative retention time; SAMS, *S*-adenosyl methionine synthetase.

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chromatography approach combined with a derivatization with phenylisothiocyanate [9–12], high amounts of tissue (100–500 mg) were required, processing times were long and results variable.

Many procedures have been developed to quantify amino acids in biological matrices (reviewed in Ref. [13]). A more recent procedure comprised of solid phase extraction (SPE), followed by immediate amino acid derivatization with methyl chloroformate and quantification with gas chromatography-mass spectrometry (GC–MS) [14] was able to quantify all amino acids except arginine (due to retention on the GC column) in plant samples, in a fast and reliable way, mainly due to the fast derivatization step, which was originally developed by Husek [15]. An overview of the reaction mechanism is given in [16]. The main advantages of this derivatization method compared with others such as trimethylsilylation, are the fast reaction time (<5 min), the mild reaction conditions (no heating required, aqueous environment allowed) and the low cost, which have led to multiple applications in different fields [16–23]. The goal of this study was to optimize and apply this method to the quantification of 19 out of 20 amino acids (all except arginine) in *A. thaliana* plant tissue, with a focus on the five amino acids (Asp, Ile, Lys, Met and Thr) that are involved in the aspartate super pathway.

2. Materials and methods

2.1. Standards and reagents

Individual amino acids (free base) and internal standard norvaline were obtained from Sigma-Aldrich (St. Louis, MO, USA). Stock standard solutions of each individual amino acid and internal standard were prepared in ultrapure water, except for asparagine (Asn) and glutamine (Gln) (in 14.5 mM NH_4OH) and for Asp and tyrosine (Tyr) (in 190 mM or 240 mM HCl respectively), based on their solubility, and stored separately at −20 °C. Standard solution mixes of amino acids were prepared according to their expected concentrations in plant tissues. All solutions, including the derivatizing agent, methyl chloroformate and pyridine were obtained from Sigma-Aldrich. A stock solution of β-estradiol was prepared in pure ethanol and stored at −20 °C. All solutions had a purity level ACS grade or higher. Ultrapure water was obtained using a Direct-Q 3 UV Type 1 (Merck Millipore, Billerica, MA, USA).

2.2. Plant material

Wild type and transgenic *A. thaliana* seedlings were all ecotype Columbia (Col-0). The *xve::dhdps-r1* line was generated via *Agrobacterium tumefaciens*-mediated floral dip transformation [24] using the pMDC7 vector [25]. The construct in this vector was produced through Gateway™ cloning (Invitrogen, Gaithersburg, MD, USA), and contains the coding sequence of a mutated *HTDPS* gene, *dhdps-r1*, which encodes an enzyme that is insensitive to feedback inhibition by Lys [9,26]. This gene is under transcriptional control of the XVE transactivator, which enables inducible expression of *dhdps-r1* by β-estradiol [27]. Seeds were surface sterilized by an incubation of 15 min in a 3/2 bleach/double distilled H_2O (ddH_2O) solution (Javel 10°, Loda, Beerse, Belgium) with 0.05% Tween-20 (Sigma-Aldrich, St. Louis, MO, USA), followed by five washes in sterilized ddH_2O. Seeds were stratified for 3–5 days in sterile ddH_2O at 4 °C in the dark before sowing.

Plants were grown in vitro on sterile K1 medium (1x Murashige & Skoog medium, 1% sucrose, 2.25 mM MES Monohydrate, 0.7% Plant Agar, pH 5.7; all components supplied by Duchefa Biochemie B.V., Haarlem, The Netherlands) in a growth room (12 h light/12 h dark cycle, 100 μmol/m^2 s^{-1}, 22 °C, 55% humidity). Filter-sterile amino acid (500 μM lysine or methionine) or sterile hormonal supplements (5 μM β-estradiol) were added after autoclaving the medium. Plants were transferred to fresh medium every week for three weeks and leaf material was harvested 22 days after sowing, weighed, frozen in liquid nitrogen and stored at −80 °C until analysis.

2.3. Solid phase extraction (SPE) and derivatization

The extraction and derivatization procedure was based on the protocol described by Menzel et al. [14] with some modifications of the solvent volumes used during SPE in order to obtain better recoveries. Using the optimized procedure (Fig. 1), 20–50 mg (fresh weight) plant material was spiked with 5 μL of a 1.25 mM norvaline (internal standard) solution and homogenized in 1 mL 0.01 M HCl with two stainless steel beads of 3 mm diameter in a TissueLyser II (Qiagen, Venlo, The Netherlands) at 25 Hz. After vortexing for 10 min and centrifugation at 14,000 *g*, the supernatant was added to a strong cation exchange solid phase extraction cartridge (Discovery DSC-SCX SPE Tube, bed weight: 100 mg, volume 1 mL (52685-U; Sigma-Aldrich, St. Louis, MO, USA)), previously activated with 1 mL 0.01 M HCl and three times 1 mL ultrapure water, using a vacuum manifold. After washing twice with respectively 1 and 0.5 mL of methanol/water (80/20, v/v), the cartridge was dried for 2 min before the amino acids were eluted with 500 μL of the elution buffer (1/1, v/v, methanol/8 M NH_4OH).

For the amino acid derivatization all steps were performed using positive displacement pipettes. Ten μL each of pyridine and methyl chloroformate were added subsequently to 100 μL of the analyte, after which the solution was mixed and incubated for 1 min to produce the derivatives (Supplemental Fig. S2). The derivatized amino acids were separated from the reaction mixture by sequential addition of 60 μL chloroform and 60 μL of a freshly prepared 50 mM sodium bicarbonate solution, followed by vortexing for 30 s and incubation for 10 s to allow phase formation. Finally, 50 μL of the bottom organic phase was transferred to a new glass tube with a few anhydrous sodium sulfate crystals. This whole cycle was repeated twice resulting in a total of 150 μL derivative solution that was finally incubated for 30 min before transfer to a micro insert in a GC–MS vial.

2.4. GC–MS analysis

Gas chromatography-mass spectrometry analyses were carried out on a Trace Ultra GC with automatic injector coupled to a quadrupole Trace MS Plus mass spectrometer (Thermo Electron Corporation, San Jose, CA, USA). Separations were obtained with a fused silica capillary column (HP5-ms, 30 mm × 25 mm, 0.25 μm, Agilent J&W, Santa Clara, CA, USA). The oven temperature was initially held at 70 °C for 3 min, raised at 25 °C/min to 280 °C, and held for 5 min. The column flow was 1.0 mL He/min. The injection volume was 4 μL (splitless mode) and the temperature of the injector was set at 240 °C. The transfer line to the mass spectrometer was kept at 290 °C, while the ion source temperature was set at 200 °C. Total analysis time was 13 min. The MS was operated in scan (50–500 *m*/*z*) mode during optimization and in SIM (selected ion monitoring) mode for quantification using the Xcalibur software. For SIM, appropriate ion sets were selected and four characteristic mass fragments of the derivatized amino acids were used for identification (Table 1).

2.5. Statistical analysis

For the qualitative interpretation, relative retention times (RRT, i.e. the ratios of the chromatographic retention time of the analyte and that of the internal standard) and identification points (IP) were calculated for all substances. Using internal QA/QC parameters, four mass fragments were selected for qualification, of which at least