Analytical Biochemistry 547 (2018) 14–18

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

Analysis of ^{13}C labeling amino acids by capillary electrophoresis – High resolution mass spectrometry for fluxomic studies

Sébastien Acket*, Anthony Degournay, Mathilde Gosset, Franck Merlier, Manual Adrian Troncoso-Ponce, Brigitte Thomasset

Sorbonne Universités, Génie Enzymatique et Cellulaire, UMR CNRS 7025, Université de Technologie de Compiègne, 60205 Compiègne Cedex, France

ARTICLE INFO

Keywords:
^{13}C labeling
Accurate assessment of labeling measurements
Amino acids
Metabolic flux analysis
Fluxomic
Flaxseed

ABSTRACT

In context of fluxomic studies, ^{13}C labeling analysis of amino acids are very important for solving the carbon flow calculation, because they are synthesized in various biosynthesis pathways and cellular compartments in plant cells. Traditionally, ^{13}C labeling analysis are performed using low resolution mass spectrometry detector by GC-MS. We developed a method using capillary electrophoresis-high resolution mass spectrometry without derivatization and with better accuracy assessment of labeling measurements comparing to classical GC-MS. Our method allowed us to show that valine, leucine, alanine are not synthesized from the same pyruvate pool during the period of reserves accumulation in flax seeds.

^{13}C-based metabolic flux analysis (^{13}C–MFA) is a powerful tool for analyzing the carbon flow in living cells [1,2]. ^{13}C–MFA describes the metabolic activity of cells, its regulation, and allows the identification of potential targets for improving biotechnological processes [1–4]. ^{13}C–MFA is performed at metabolic steady state using isotope labeling measurements. *In vivo* carbon fluxes are calculated from labeling measurements using a model-based approach that maximize the best fit between experimental measures and model-predicted labeling distributions. Labeling measurements can be obtained either in the stationary isotopic steady-state (MFA standard) or in the non-stationary isotopic steady-state (INST–MFA) [4–6]. Amino acids labeling measurements is crucial for solving carbon flow calculation, especially in plant cell, where carbon metabolism is compartmentalized. Plant glycolysis is located in the cytosol and within plastids [7] and the majority of amino acids are synthesized in these latter subcellular organelles and therefore reflecting plastidic metabolism [8]. Currently, amino acid positional isotopomers are quantitatively determined by ^{1}H NMR and ^{13}C NMR [9,10], whereas the number of carbon isotopic atoms (^{13}C) incorporated (mass isotopomer) is traditionally measured using a method based on gas chromatography coupled to a low resolution mass spectrometer (GC–MS) in full scan mode. Quantification of *in vivo* carbon fluxes at high resolution is essentially dependent on accurate assessment of labeling measurements. Measure of mass isotopomers corresponds to the ratio of the peak areas of the isotopic distribution (m0, m + 1, …, m + n) on a fragment of a molecule containing n carbon, corrected by the natural abundance [8,11–14]. GC-MS method requires derivatization of the amino acids to their *N,O-tert*-butyldimethylsilyl derivatives (TBDMS). It is a robust and sensitive method for the quantification of all amino acids, used for many years [15], but presented several drawbacks determining of amino acids ^{13}C labeling due to the necessity of amino acids derivatization. It is time consuming due to sample preparation with low stability of derivatized amino acids, and requires precautions to ensure that all amino acids are derivatized. Derivatizing agent adds exogenous carbon atoms that have a natural abundance of 1.07% ^{13}C and thereofore perturbing the amino acids ^{13}C labeling. This complicates the data analysis and it is can be source of error [16]. Moreover, isotopic distribution peaks of characteristic fragments can be diluted by other isotopic distribution fragments peaks from the same molecule. Low-resolution mass analyzers are unable to differentiate these peaks and are prone to generating errors during the measure of the isotopic distribution pattern [16].

In this work we propose the use of capillary electrophoresis (CE) coupled to high-resolution mass spectrometry (HRMS) as a method that simplifies and overcomes the previously described technical limitations associated to GC–MS during the analysis of ^{13}C labeling amino acids for fluxomic applications. CE allows us a simple sample preparation, without derivatization (gain of time, sample stability, and no addition of exogenous carbon atoms that could induce errors of labeling measurements) [17–19]. HRMS in full scan mode allows measuring the ratio of the peak areas of the isotopic distribution with better accuracy assessment of labeling measurements. It is accomplished directly from the quasi-molecular ion $[M+H]^+$ of each amino acids containing all

* Corresponding author.
E-mail address: sebastien.acket@utc.fr (S. Acket).

https://doi.org/10.1016/j.ab.2018.02.009
Received 9 November 2017; Received in revised form 7 February 2018; Accepted 8 February 2018
Available online 13 February 2018
0003-2697/

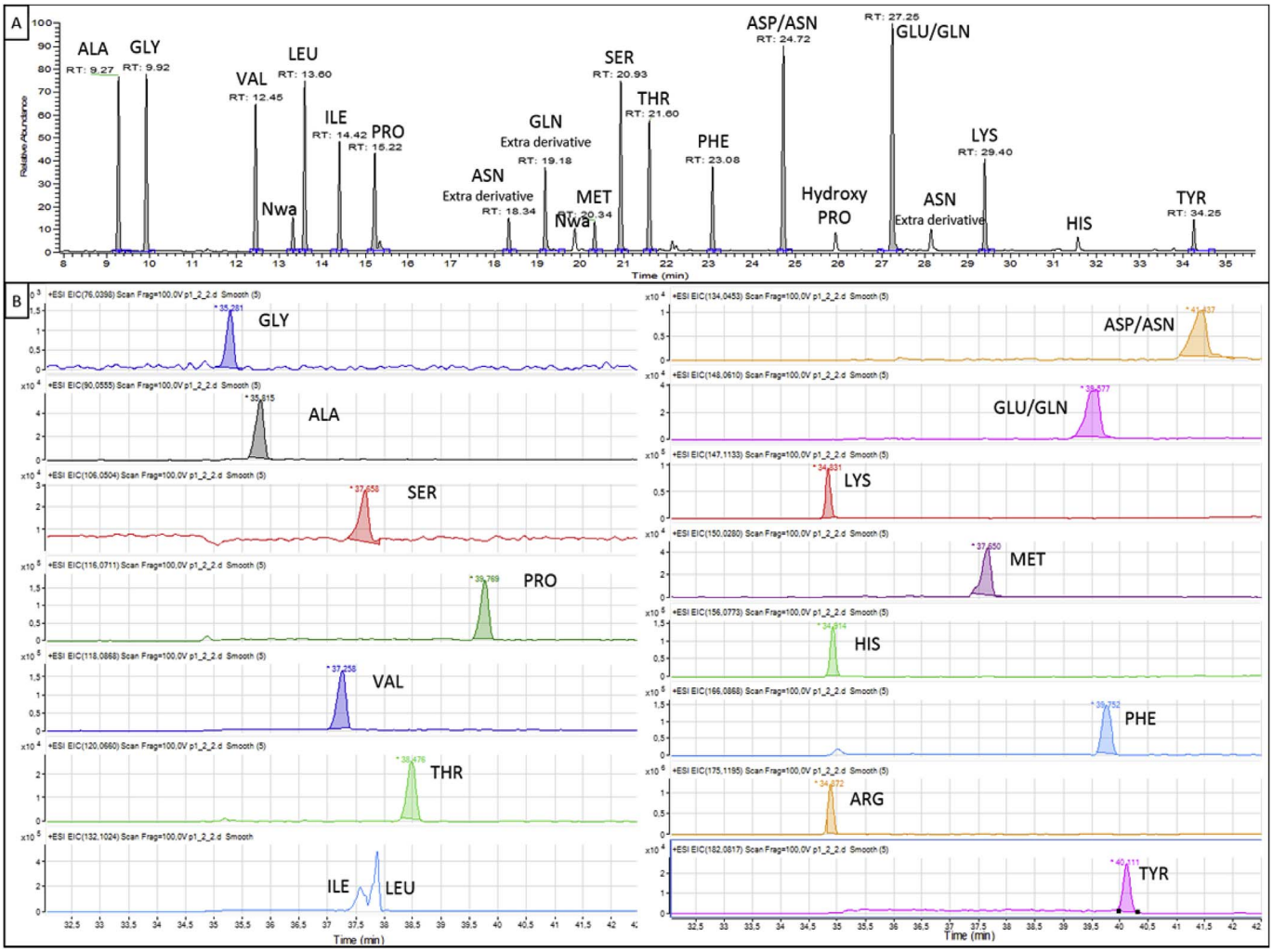

Fig. 1. GC–MS chromatograms for TBDMS free amino acids (A) and Selected CE–HRMS electropherograms for free amino acids (B) in flaxseed embryos collected at 16DAF. The numbers on the right indicate the exact mass of each amino acid obtained by this analysis.
Abbreviations: ALA: Alanine; GLY: glycine; VAL: Valine; Nwa: Artifact of derivatization; LEU: leucine; ILE: Isoleucine; PRO: Proline; ASN: Extra derivative of Asparagine; GLN: Extra derivative of glutamine; MET: Methionine; SER: Serine; THR: Threonine; PHE: Phenylalanine; ASP/ASN: mixture of aspartate and asparagine; GLU/GLN = mixture of glutamate and glutamine; Hydroxy PRO: Hydroxyproline; LYS: Lysine; HIS: Histidine; TYR: Tyrosine.

the carbon atoms of the molecule (no fragment and exogenous carbon). The assessment of both experimental approaches, GC–MS and CE–HRMS, was illustrated with the data obtained by the analysis of isotopic natural abundance of free amino acids extracted from flax (*Linum usitatissimum* L.) embryos. Accuracy assessment of isotopic natural abundance was measured by both methods comparing the ratio of the peak areas of the isotopic distribution (m0, m + 1, …, m + n) of the experimental natural isotopic distribution abundance of amino acids extracted from flaxseed with the theoretically expected values simulated using an isotope distribution calculator. Due to a better accuracy assessment, CE–HRMS was used for measuring ^{13}C isotopic enrichments of amino acids extracted from flaxseed embryos incubated with carbon-labeled substrates (^{13}C).

Flax plants were grown in greenhouse, seeds were harvested at 16 days after fertilization (16 DAF), sterilized, and 10 embryos extracted from seeds were incubated in a Petri dish containing 16 mL of an optimized culture medium comprising substrates (80 mM unlabeled glucose, 20 mM [U-^{13}C] glucose, 640 mgL^{-1} unlabeled glutamine, 160 mgL^{-1} [U-^{13}C] glutamine) and macro elements as previously described [16]. Embryos were cultured under controlled conditions as described by Acket et al., 2017 [16]. After 24, 72, 120, and 168 h of incubation with ^{13}C substrates embryos were harvested and washed 3 times with 10 mL of sterile water. The extraction method selected was a standard sequential extraction protocol widely used by the metabolic flux plant community [4–6] and optimized for flaxseed embryos allowing a maximum metabolite extraction [16]. Primary and secondary soluble compounds (free sugars, amino acids, organic acids, sugars phosphate, and polyphenols) were extracted with 1 mL of 0.01% HCl in a water bath at 95 °C during 15 min. This procedure was repeated 3 times, to maximize the extraction of metabolites. After lyophilization, the soluble molecules were resuspended in 1 mL of sodium acetate buffer (50 mM, pH 5.5) and loaded onto a cation exchange resin (Dowex 50 × 8 [Hydrogen form, 200–400], Sigma-Aldrich 217514). The purified amino acids were then frozen with liquid nitrogen and lyophilized. The lyophilizate was resuspended in 500 µL of water and directly injected into CE–HRMS, or derivatized for GC–MS analysis (the derivation procedure used is described in ref [8], [11–13]).

Amino acids samples extracted from flaxseed embryos and commercial standards were analyzed by GC–MS and CE–HRMS. For GC–MS analysis, chromatographic separation was performed using a Thermo Scientific GC–MS (GC-Quantum) instrument coupled with an Equity 5 ms capillary column (5% phenyl-methyl-siloxane/95% diphenylpolysiloxane), 30 m × 0.25 mm, 0.25 µm) after derivatization of the amino acid pool to their TBDMS derivates. The chromatographic separation conditions were summarized in Table S1 and the mass spectrometer conditions in Table S2. The measurements of the isotopic