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NMR-based metabolomics study of the biochemical relationship between sugarcane callus tissues and their respective nutrient culture media

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Abstract

The culture of sugarcane leaf explant onto culture induction medium triggers the stimulation of cell metabolism into both embryogenic and non-embryogenic callus tissues. Previous analyses demonstrated that embryogenic and nonembryogenic callus tissues have distinct metabolic profiles. This study is the follow-up to understand the biochemical relationship between the nutrient media and callus tissues using one-dimensional (1D ^1H) and two-dimensional (2D ^1H – ^{13}C) NMR spectroscopy followed by principal component analysis (PCA). 1D ^1H spectral comparisons of fresh unspent media (FM), embryogenic callus media (ECM), non-embryogenic callus media (NECM), embryogenic callus (EC), and non-embryogenic callus (NEC), showed different metabolic relationships between callus tissues and media. Based on metabolite fold change analysis, significantly changing sugar compounds such as glucose, fructose, sucrose, and maltose were maintained in large quantities by EC only. Significantly different amino acid compounds such as valine, leucine, alanine, threonine, asparagine, and glutamine and different organic acid derivatives such as lactate, 2-hydroxyisobutyrate, 4-aminobutyrate, malonate, and choline were present in EC, NEC, and NECM, which indicates that EC maintained these nutrients, while NEC either maintained or secreted the metabolites. These media and callus-specific results suggest that EC and NEC utilize and/or secrete media nutrients differently.

Keywords

MS3DC medium; Embryogenic callus; Non-embryogenic callus; NMR; Fold change; Pathway analysis

Introduction

Plant regeneration through somatic embryogenesis has been reported in sugarcane using young leaf rolls and immature inflorescences [1]. Somatic embryogenesis is a process where, under the appropriate environment, somatic cells develop into embryos which structurally and functionally resemble zygotic embryos [2]. The factors controlling this process have been studied extensively in many plant species [3–6].

It has been shown that callus type is related to specific metabolites, for example, somatic embryogenesis in coconut calluses were linked to specific higher uptake of dry matter of NH_4^+ , Ca^{2+} , Mg^{2+} , and sucrose from the medium [7]. EC has more carbohydrate and starch content than NEC [8]. Comparative analysis of zygotic and somatic embryogenesis of *Acca sellowiana* showed higher amounts of sucrose and fructose [9]. In *Picea abies*, glucose, fructose, and sucrose were detected in somatic embryo [10]. High carbohydrate concentration seemed to play a critical role in embryogenesis efficiency and embryo development [11]. Somatic embryo-genesis in *Hevea* is stimulated when the embryogenesis induction medium contains maltose [12]. Induction of somatic embryogenesis in the presence of glucose, fructose, or sucrose revealed strong callus growth in the first 3–4 weeks, associated with a high intra-extracellular hexose content and a high starch content [13]. Higher concentrations of sucrose, glucose, and fructose were also identified in EC of sugarcane whereas lower concentrations of those metabolites were identified in NEC [14].

The development of EC of sugarcane and cell suspensions is related to the type and amount of intracellular proteins in the callus cells and to the secreted proteins from these cells into the medium [15]. Embryogenic or organogenic calli subcultured onto shoot differentiation medium triggers the stimulation of cell metabolism principally at three levels namely (i) initiation of photosynthesis, glycolysis, and phenolic compounds synthesis; (ii) amino acid-protein synthesis, and protein stabilization; (iii) sugar degradation [16].

It is clearly understood that sugarcane tissues from young leaves cultured in vitro in the presence of 2,4-dichlorophenoxyacetic acid (2,4-D) induces two callus types: a white (embryogenic) and mucilaginous callus (nonembryogenic) [17]. These callus types can be separated to establish embryogenic and non-embryogenic callus metabolic profiles [14]. However, the relationship between callus tissues and their media at the biochemical level such as nutrient uptake by callus from medium or release of chemicals to the medium is not well understood or has not been explained.

Analysis of the metabolic profiles could shed some light on a biochemical relationship between callus tissues and their media. It is commonly accepted that an analytical technique alone will not provide sufficient information about the metabolic profile and, therefore, must be used in conjunction with other techniques for a comprehensive view [18]. NMR spectroscopy offers a wide spectrum chemical analysis technique, which is rapid, non-destructive, reproducible, and offers basic sample preparation and sample stability. At the same time, multivariate statistical analysis such as principal component analysis (PCA) has been designed to analyze complex data [18]. The NMR-based metabolomics approach offers a non-targeted, quantitative identification of the metabolites present in a sample and can

reveal unexpected properties of model and non-model systems that result from cellular adjustments to stressors [19, 20]. Metabolic profile analyses in plant biotechnology research using ^1H NMR was coupled with principal component analysis (PCA) and has recently been described [16, 21].

This study reports on metabolic profile analysis using NMR spectroscopy coupled with PCA, a potential tool to investigate crucial information that points to a biochemical relationship between media and callus tissues. The aim of this study was to compare metabolic profiles of FM, EC, ECM, NEC, and NECM and to identify potential metabolites related with nutrient media and callus tissues at the biochemical level.

Materials and methods

Sample preparation and harvesting

Callus tissue was induced, maintained, and harvested. For media samples (MS-3DC), 200–300 mg of fresh media (FM) underneath each type of callus was sampled: embryo-genic callus media (ECM) and non-embryogenic callus media (NECM). Squares of media were removed from underneath the calli, dipped in liquid nitrogen, and lyophilized overnight to remove the water content.

Metabolite extraction

The dried FM, EC, ECM, NEC, and NECM were subjected to methanol-chloroform-water extractions as described by Kim et al. [22]. Briefly, 20 mg of dried sample was used for each sample/replicate (6 replicates per sample). Solvent volumes were calculated at a constant ratio of 2:2:1.8 of chloroform:methanol:water according to the Bligh and Dyer method [23, 24] using the dry mass. Centrifugation was applied to achieve two liquid layers separated by a solid protein layer. The hydrophilic metabolite extracts from the top layer were dried with vacuum and centrifugal force using a Centrivap centrifuge for ~15 h. The hydrophobic extracts from the bottom layer were stored at -80°C for future analysis.

NMR sample preparation and spectroscopy

The dried hydrophilic extracts from each sample were re-suspended in 620 μL of NMR buffer (100 mM sodium phosphate buffer (pH 7.3), 1 mM TMSP (internal standard, 3-(trimethylsilyl) propionic-2,2,3,3- d_4 acid, CAS: 24493-21-8), and 0.1 % sodium azide, in 99.9 atom % D_2O). All experiments were conducted at 298 K using 600 μL of sample in 5 mm NMR tubes (Norell). The spectra were recorded on a BrukerAvanceTM III spectrometer operating at 700 MHz. The first increment of a presat-noesy experiment was acquired for each sample using the Bruker noesypr1d pulse sequence. All data were collected using a spectral width of 16.0 ppm and 64 K points resulting in an acquisition time of 2.9 s; on-resonance pre-saturation was used for solvent suppression during a 3-s recycle delay. Each experiment was collected with 120 scans, 4 dummy scans, 3 s relaxation delay, and pre-saturation at the residual water frequency. The 90° pulse widths were measured for each sample using the automatic pulse calculation experiment (pulsecal) in TopSpin 2.1.1 (BrukerBioSpin, Billerica, MA). Two-dimensional ^1H – ^{13}C HSQC data were collected for a representative EC, NEC, NECM, ECM, and FM sample using a Bruker hsqcedetgpcsp2.2

pulse sequence. The ^1H was observed in the F2 channel with a spectral width of 11 ppm while the ^{13}C was observed in the F1 channel with a spectral width of 180 ppm.

NMR data analysis

Multivariate statistical analysis—Bucket tables were generated by using AMIX software (version 3.9.7, BrukerBioSpin, Billerica, MA). Bucket intensities were normalized to the total intensity and spectra were binned into 0.01 ppm wide buckets over a spectral region of 0.5–10.0 ppm using advanced bucketing in AMIX. From each spectrum, the water region (4.75–4.90 ppm) was eliminated. PCA was performed on the bucket tables generated from AMIX using both AMIX and MetaboAnalyst 2.0 (MetaboAnalyst 2.0—a comprehensive server for metabolomic data analysis) [25]. For each scores plot generated during the analysis, Mahalanobis distance (D_M), two-sample Hotelling's T^2 statistic (T^2), F values (F_t) and critical F values (F_c) were calculated using MatLabR2010b [26].

Spectral analysis and metabolite identification—In order to identify the metabolites that are different when comparing embryogenic and non-embryogenic callus tissue and media, fold changes, and related p values were determined for each bucket. Statistically significant changing buckets for pair-wise analysis (FM vs EC/NEC/NECM/ECM) were determined using both AMIX and MetaboAnalyst 2.0. The significant bucket intensity values identified by AMIX ($p < 0.011875$, 0.021591 , 0.0084821 , and 0.0095 , respectively; Bonferroni-corrected confidence intervals for significant metabolite analysis) were exported to Microsoft Excel and fold changes were manually calculated by subtracting two corresponding buckets [27]. Calculations of fold changes and T tests were also performed with MetaboAnalyst 2.0 in order to determine whether or not the changes in bucket intensities were statistically significant. Buckets with corresponding p values less than 0.05 were considered statistically significant. When comparing the significant buckets identified by both AMIX and MetaboAnalyst 2.0, there was an 84 % agreement. The 16 % of buckets that were identified by only one software was also included. The metabolites corresponding to these peaks in the buckets were identified using the Biological Magnetic Resonance Data Bank (BMRB) (<http://www.bmrwisc.edu/metabolomics/>), Madison Metabolomics Consortium Database (MMCD) (<http://mmcd.nmrfa.wisc.edu/>), and Chenomx NMR Suite (Chenomx Inc., Edmonton, Alberta, Canada). Metabolic data interpretation was facilitated with the Metabolic Pathway Analysis (MetPA) component of MetaboAnalyst 2.0 [25].

Results and discussion

1D ^1H NMR spectral comparisons

Stacked 1D ^1H NMR spectra (0.85–8.5 ppm) of FM, EC, ECM, NEC, and NECM were analyzed, with expansions of the lower intensity aliphatic (0.8–3.19 ppm), sugar (3.34–5.40 ppm), and aromatic (5.9–8.5 ppm) regions. Metabolic profile differences among the spectral profiles of FM, EC, ECM, NEC, and NECM are clear (Fig. 1) and can be used to determine the biochemical relationship between media and callus.

Consumption of nutrients from media—There are 11 metabolites present in FM. These are acetate, glutamine, proline, succinate, glucose, fructose, sucrose, maltose,

tyrosine, phenylalanine, and formate (labeled in Fig. 1 and listed in Table 1). Of these metabolites, glutamine, proline, tyrosine, and phenylalanine were absent in both ECM and NECM but present in both EC and NEC callus. Acetate was absent in NECM and both types of calluses, while succinate was absent from NEC and NECM. Glucose, fructose, sucrose, maltose, and formate were present in all media and calluses. The variation of metabolites (originating from FM) in the spent media (ECM and NECM) and calluses (EC and NEC) indicates that the metabolites were consumed differently by each type of callus.

Utilization and secretion of nutrients by callus—Figure 1 and Table 1 show that metabolites such as threonine, lactate, and unknown peak (UP)-04 were present in ECM, EC, and NEC but absent in FM and NECM, which indicates that these metabolites may have been secreted from EC to the media but have been completely utilized by NEC. On the other hand, 4-aminobutyrate, aspartate, asparagine, putrescine, ethanolamine, malonate, and pyridoxine were present in NECM, EC, and NEC, but absent in FM and ECM, and suggests that these metabolites may have been secreted from NEC to NECM but could have been maintained or utilized by EC. 2-Hydroxyisobutyrate, UP-01 and UP-03 were present in ECM, NECM, EC, and NEC but absent in FM, indicating that these metabolites may have been secreted from EC to ECM and NEC to NECM. Glucose, fructose, sucrose, maltose, and formate were present in all media and calluses, which indicate that those metabolites were supplied in excess quantity and therefore may have been left over and present in both the calluses and media. Allantoin is a sugar derivative, present only in NEC and NECM, which possibly indicates that sugar was converted to allantoin and then secreted to the medium. Qualitative differences in metabolites in both callus tissues and their corresponding media indicate that metabolites can be utilized, maintained, and secreted differently by calluses. Several metabolites such as, valine, leucine isoleucine, alanine, lysine, β -alanine, choline, uridine, histidine, tryptophan, thymidine, guanosine, adenine and UP-02 were absent in all three media but present in both types of calluses.

Proton (^1H) NMR spectroscopy is a particularly good choice in plant metabolomics studies given the universal occurrence of protons in organic metabolites. The main disadvantages of this analytical method are the low sensitivity, high spectral complexity, and peak overlap. Due to the extensive signal overlap, ^1H - ^{13}C (HSQC) correlation experiments were also recorded (data not shown) in order to separate some of the signals in the ^{13}C dimension. Based on these spectral metabolic profile comparisons, it can be speculated that different callus tissues and their nutrient media have varied biochemical relationships. Also, the spectral profile in the aliphatic, sugar, and aromatic region changes significantly (see labeled peaks in Fig. 1). For understanding this biochemical relationship, metabolic fold change analysis was carried out.

Determination of significant metabolites fold changes

Based on their fold change analysis, among the 39 identified metabolites from 1D ^1H NMR spectra, 15 metabolites were significantly related with callus tissues and their nutrient media when ^1H NMR spectral profiles of FM were compared with each EC/ECM/NEC/NECM. The sugar metabolites, glucose, fructose, sucrose, and maltose were identified as significantly higher in concentration in FM and EC only (Fig. 2a and Table 2). The amino

acid metabolites, valine, leucine, and alanine were identified as significantly higher in concentration in EC and NEC only; however, NEC has higher level than EC. Threonine was identified as significantly higher in concentration in EC and NECM, and NECM has higher level than EC. Asparagine and glutamine were identified as significantly higher in concentration in NEC and NECM only (Fig. 2b and Table 2). The organic acid derivatives, lactate and 4-aminobutyrate were identified as significantly higher in concentration in EC, NEC, and NECM, but NEC and NECM had higher levels. 2-Hydroxyisobutyrate was identified as significantly higher in concentration in NEC and NECM. Malonate was identified as significantly higher in concentration in NEC, and choline was identified as significantly higher in concentration in EC and NEC, where NEC has the higher level (Fig. 2c and Table 2, inserted in Fig. 2).

Metabolic pathways analysis—Metabolic pathway analysis was performed using the significantly changing metabolites identified by PCA-based fold analysis. Pathway analysis suggested the involvement of 18 different significant metabolic pathways based on p values < 0.05 and an impact factor threshold greater than zero. Metabolic profile analysis of FM, EC, ECM, NEC, and NECM revealed that biochemical relationships between callus tissues and their nutrient media regulate an array of metabolites involved in pathways. Levels of sugars, amino acids, and different aliphatic compounds belonging to starch and sucrose metabolism, glycolysis/ gluconeogenesis, alanine, aspartate, and glutamate metabolism, pyruvate metabolism, glycine, serine, and threonine metabolism, aminoacyl-tRNA biosynthesis, TCA cycle, and valine, leucine, and isoleucine biosynthesis and degradation pathways, changed significantly in response to different biochemical relationships between callus tissues and their nutrient media (Fig. 3).

Discussion

1D ^1H spectral comparisons

This 1D ^1H NMR-based metabolomics profile study with biochemical pathway analysis was conducted to understand the changes of metabolite pattern among different calluses and their nutrient media. 1D ^1H spectral comparisons of different calluses and their nutrient media showed distinct metabolic profile differences. It has been shown that 1D ^1H NMR spectroscopy can be used to compare the inner and outer cells of *Catharanthus roseus* calli [28]. By visual inspection, ^1H NMR signal intensity can be used for comparing healthy and infected *C. roseus* leaves [29]. Embryogenic and non-embryogenic callus tissues of sugarcane were compared using 1D ^1H NMR to understand the metabolic profile differences between them. A broad range of compounds such as amino acids, carbohydrates, organic acids and phenolic compounds from callus tissue were identified and analyzed by ^1H NMR [16]. However, a typical proton NMR spectrum of plant materials contains thousands signals [22] and extensive peak overlap [30]. The ^1H assignments of these studies were verified using 2D ^1H - ^{13}C HSQC spectral data. In order to verify the extensive peak overlap, 2D ^1H - ^{13}C correlation experiments were conducted to clearly understand the spectral information [30]. Similar to the work described in these reports, the results presented here using 1D ^1H NMR for comparing metabolic profiles of callus tissues and their nutrient media can be useful for understanding the biochemical relationships among them.

Metabolite fold change and the relationship with biochemical pathways

In this study, based on fold change analysis, different sugar metabolites such as glucose, fructose, sucrose, and maltose were identified as significantly higher in concentration in FM and EC only, which indicated that during embryo formation EC uptakes and maintains sugar nutrients from the media while NEC utilized/underutilized sugar nutrient from the media in which they may have been converted into different metabolites such as amino acids and organic acids. In a previous sugarcane callus study, different sugars such as sucrose, glucose, and fructose were identified with higher levels in EC whereas with lower levels in NEC [14]. Initiation of embryogenesis in coconut plant was linked with higher uptake of sugar compounds from the callus media [7]. In this study, biochemical pathway analysis suggested that EC can be interconnected with gluconeogenesis and starch, sucrose metabolism pathways for uptaking and/or synthesizing more sugar compounds for maintaining the embryo, whereas, in NEC, those sugar synthesizing pathways can produce the precursors for either amino acid or organic acid metabolism or degradation pathways through pyruvate metabolism (Fig. 3). The results explained in this manuscript are in agreement with all of these reported studies in that the uptake, utilization, and maintenance of sugars by EC may be involved with somatic embryogenesis rather than releasing of metabolites to the media, which explains the absence of sugars in ECM. However, in NEC, since no somatic embryos are formed, these sugars are not stored rather converted to other amino acids or organic acids which are secreted and are present in NECM.

Different amino acid metabolites such as valine, leucine, and alanine were identified as significantly higher in concentration in EC and NEC; however, NEC has higher levels than EC, which suggests that during embryo formation EC utilizes more amino acid content than NEC. Threonine was identified as significantly higher in concentration in EC and NECM, where NECM has higher level than EC, suggesting that instead of utilizing threonine, NEC secreted it. Asparagine and glutamine were identified as significantly higher in concentration in NEC and NECM, which also points to underutilization and secretion of these amino acids. In ECM, no amino acid metabolites were found as significantly higher in concentration. This clearly indicates that EC does not release amino acid metabolites to the media. During somatic embryo formation, decreased levels of protein contents were reported in soybean [31] and cumin [32]. In comparing embryogenic and non-embryogenic callus tissue of sugarcane, significantly lower levels of amino acids such as asparagine, glutamine, and lysine, and also higher levels of alanine, were identified using 1D ^1H NMR spectroscopy. In the callus maintenance medium, different nitrogen sources such as L-proline, L-asparagine and casein hydrolysate marked an effect on wheat somatic embryo formation [33]. Based on biochemical pathway analysis (as seen in Fig. 3), it can be speculated that in EC, alanine, valine, and leucine can act as possible precursors to carbohydrate synthesis through pyruvate metabolism and in NEC, alanine, valine, leucine, threonine, asparagine, and glutamine may be underutilized or can be converted to different organic acids through valine, leucine, and isoleucine metabolism/ degradation pathways or may have been released to the media. These reports imply that amino acids that NEC uptakes and possibly underutilizes may be converted to other metabolites, remain the same in NEC, or be released to the media. Similarly with sugars, EC uptake, utilization, and maintenance of amino acids may be involved with somatic embryogenesis.

In this study, organic acid derivatives such as lactate and 4-aminobutyrate were identified as significantly higher in concentration in EC, NEC, and NECM, where NEC and NECM had higher levels, indicating that higher levels of these compounds are related with embryo dormancy or stress adaptation. 2-Hydroxyisobutyrate was identified as significantly higher in concentration in NEC and NECM, which also points to embryo dormancy. Malonate was identified as significantly higher in concentration in NEC. Malonate is an important metabolite that contributes during respiratory activity of the dormant tuber of potato [34]. Choline was identified as significantly higher in concentration in EC and NEC where NEC has higher levels, which could link this compound to callus tissue stress adaptation. Endogenous regulation of embryo dormancy may be regulated due to the presence of higher level of different organic acids [14]. Choline and 2-hydroxyisobutyrate were identified as significantly decreased in concentration in EC when compared to NEC of sugarcane. Explant pretreated with different organic acids such as citric acid, ascorbic acid, ascorbic acid/citric acid, and tri-potassium citrate/citric acid resulted in decreased somatic embryo formation [35]. Plant stress adaptation is related with increasing concentration of choline [36]. These reports and the results presented here suggest that in EC, uptake, utilization and maintenance of different organic acids and choline may be involved with somatic embryogenesis, however NEC uptakes and underutilizes these compounds by converting them to other metabolites or discharging them.

Conclusion

It is clear that 1D ^1H NMR-based spectral comparison can be a useful tool for understanding the distinct metabolite differences among FM, EC, ECM, NEC, and NECM. Data in this research suggested that biochemical relationships between callus tissues and their nutrient media are distinctly different in direction, i.e., EC uptakes, utilizes, and maintains nutrients from the media for embryogenesis; however, NEC uptakes, underutilizes, and degrades nutrients by converting them to other metabolites or secreting them to the media. The evidence presented here concerning the biochemical relationships between calluses and media indicates that EC and NEC utilize nutrients from their culture media differently.

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Fig. 1. Stacked 1D ^1H NMR spectra of FM, EC, ECM, NEC, and NECM of sugarcane leaf explants, with enlargements of the aliphatic (0.8–3.19 ppm), sugar (3.34–5.40 ppm) and aromatic (5.5–8.5 ppm) regions. Spectral profile showing **a** aliphatic region, **b** aliphatic and sugar region, **c** sugar region, and **d** aromatic region. The spectral profile among FM, EC, ECM, NEC, and NECM changes significantly in the sugar and aliphatic regions. UP 01–04=unknown peak regions

Fig. 2.

Fold changes (FC) for metabolites determined to be significantly changing when comparing FM and EC/ECM/NEC/NECM based on scores and volcano plots. Fold changes >1.00 indicate an increase in metabolite concentration and those with FC<1 indicate decrease in metabolite concentration. **a** Significantly changing sugar metabolites, **b** significantly changing amino acid metabolites, and **c** significantly changing organic acid derivatives

Fig. 3. Metabolic pathways leading to synthesis of metabolites. KEGG database and MetaboAnalyst 2.0 were used to elucidate metabolic networks. The metabolites that accumulated, and were identified as significantly changing in concentration by NMR, are shown in *bold*

Table 1

List of identified metabolites and unidentified peaks. Metabolites series 1–21 were identified differently from the aliphatic region, 22–26 were from the sugar regions, and 27–39 were from the aromatic region of 1D ^1H NMR spectral comparisons of FM, EC, ECM, NEC, and NEC media. Unknown peaks (UP)-1, 2, and 3 were identified from the aliphatic region and UP-4 was identified from the aromatic region of the same spectral comparisons

	List of identified metabolites	FM	ECM	NECM	EC	NEC	^1H chemical shifts (ppm)
1	Valine	–	–	–	✓	✓	0.98, 1.03
2	Leucine	–	–	–	✓	✓	0.94, 0.95
3	Isoleucine	–	–	–	✓	✓	1.00
4	Threonine	–	✓	–	✓	✓	1.32
5	Lactate	–	✓	–	✓	✓	1.32
6	2-Hydroxyisobutyrate	–	✓	✓	✓	✓	1.34
7	Alanine	–	–	–	✓	✓	1.47
8	Lysine	–	–	–	✓	✓	1.72
9	Acetate	✓	✓	–	–	–	1.90
10	4-Aminobutyrate	–	–	✓	✓	✓	1.89, 2.28, 3.00
11	Methionine	–	–	✓	✓	✓	2.63
12	Glutamine	✓	–	–	✓	✓	2.45
13	Proline	✓	–	–	✓	✓	2.34, 3.33
14	Succinate	✓	✓	–	✓	–	2.39
15	β -Alanine	–	–	–	✓	✓	2.54, 3.17
16	Aspartate	–	–	✓	✓	✓	2.80
17	Asparagine	–	–	✓	✓	✓	2.94, 2.84
18	Putrescine	–	–	✓	✓	✓	1.76, 3.04
19	Ethanolamine	–	–	✓	✓	✓	3.13
20	Malonate	–	–	✓	✓	✓	3.12
21	Choline	–	–	–	✓	✓	3.19
22	Glucose	✓	✓	✓	✓	✓	5.22, 4.63, 3.24
23	Fructose	✓	✓	✓	✓	✓	4.01, 3.69
24	Sucrose	✓	✓	✓	✓	✓	5.40, 4.20, 4.04
25	Maltose	✓	✓	✓	✓	✓	5.22, 4.63, 3.84
26	Allantoin	–	–	✓	–	✓	5.38
27	Uridine	–	–	–	✓	✓	5.89–5.91
28	Fumarate	–	–	–	✓	–	6.51
29	Tyrosine	✓	–	–	✓	✓	7.18, 6.89
30	Histidine	–	–	–	✓	✓	7.08
31	Phenylalanine	✓	–	–	✓	✓	7.32–7.42
32	Tryptophan	–	–	–	✓	✓	7.53
33	Thymidine	–	–	–	✓	✓	7.64
34	Pyridoxine	–	–	✓	✓	✓	7.66
35	Guanosine	–	–	–	✓	✓	7.98

	List of identified metabolites	FM	ECM	NECM	EC	NEC	¹ H chemical shifts (ppm)
36	Adenine	–	–	–	✓	✓	8.23
37	AMP	–	–	–	–	✓	8.24
38	ADP	–	–	–	–	✓	8.25
39	Formate	✓	✓	✓	✓	✓	8.44
40	Unknown peaks (UP)-01	–	✓	✓	✓	✓	1.12–1.31
41	UP-02	–	–	–	✓	✓	2.5
42	UP-03	–	✓	✓	✓	✓	2.64–2.76
43	UP-04	–	✓	–	✓	✓	4.35–4.61