A comprehensive method for in-depth profiling of secreted metabolites in cell culture media from human adipose stem cells

Monitor over 110 cell culture media components using the SCIEX 7500 system

Marialuce Maldini[1], Valentina Bina[2], Giulia Gastaldi[3] and Eshani Nandita[3]
Biopharma
[1]SCIEX, Italy
[2]Department of Molecular Medicine, University of Pavia, Italy
[3]SCIEX, USA

This technical note demonstrates a sensitive profiling method for analysis of secreted metabolites in cell culture media. Over 110 cell culture metabolites were monitored in under 20 min using a single method featuring fast polarity switching on the SCIEX 7500 system. Statistically significant quantitative trends demonstrated which components were upregulated or downregulated over the cell growth stages.

Human adipose derived stem cells (hASCs) are multipotent cells capable of differentiating into multiple cell lineages. Unique ability of hASCs to specialize into specific cell types when placed under select experimental conditions has increased their functional importance in regenerative medicine. hASCs secrete various metabolites that can affect cellular function and activity. Therefore, secretome analysis of hASCs has become a key procedure in the development of cell and/or tissue based therapeutics for regenerative medicine to monitor their therapeutic potential.[1]

Common approaches for the secretome analysis, such as, NMR and IR are unable to provide a comprehensive analysis of the secreted metabolites. Considering the multitude of possible secreted metabolites, LC MS/MS provides a strong solution with high sensitivity, selectivity, speed and robustness enabling profiling of multiple analytes using a single analytical platform.[2]

Herein, a comprehensive profiling of cell culture metabolites secreted from hASCs was performed using a highly sensitive LC-MS/MS workflow (Figure 1). Over 110 cell culture analytes were monitored under a 20 min run time using the SCIEX 7500 system.

Key features of the cell culture media analysis workflow for secreted metabolite profiling on the SCIEX 7500 system

- Achieve sensitive profiling of over 110 cell culture metabolites secreted from hASCs in under a 20 min run time
- Utilize a single platform method with fast polarity switching for comprehensive analysis of metabolites in positive and negative modes using the SCIEX 7500 system
- Deduce statistically significant quantitative trends from secreted metabolites to demonstrate cell growth behavior at different stages using MarkerView software with t-test and partial least squares-discriminant analysis (PLS-DA)
- Easily acquire, process and manage data using a streamlined interface using the SCIEX OS software

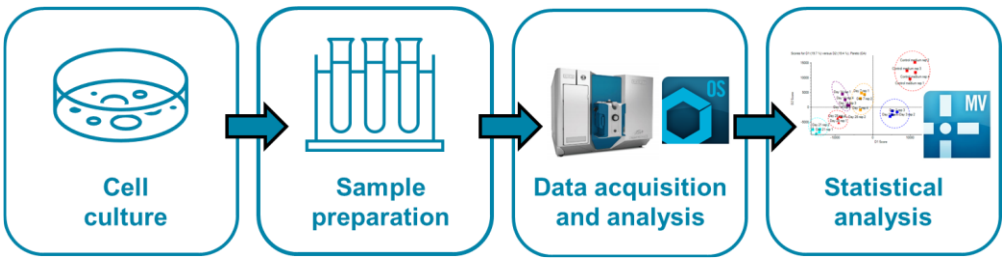

Figure 1. Workflow for the monitoring of secreted metabolites from hASCs. hASCs were cultured over the course of 28 days where analysis at day 0, 1, 3, 7, 14, 21 and 28 was performed using SCIEX 7500 system and analyzed using SCIEX OS software. For statistical analysis, MarkerView software was employed.

Methods

Figure 2. SCIEX cell culture media analysis (CCMA) method contains > 110 media components with under a 20 min analysis. The method is composed of target analytes from classes such as, amino acids, vitamins, carbohydrates, fatty acids, nucleic acids and other essential components.

Sample preparation: The hASCs were grown in cell culture medium and collected as following day 1, 3, 7, 14, 21 and 28. The hASCs were cultured in cell culture medium up to 95% confluence. After 3 expansion cycles, the cells were trypsinized, centrifuged and cultured up to 28 days as 3D pellets. Media samples were diluted in 100 µL of 50:50 (v:v) 0.1% formic acid (FA) in water and 0.1% FA in acetonitrile and centrifuged. The supernatant was diluted 60-fold with 0.1% FA.

Chromatography: Analytes were separated using a Phenomenex Kinetex F5 column (150 mm x 2.1 mm, 2.6 µm, 100 Å). Total method time was 20 min at a flow rate of 200 µL/min. Mobile phase A was composed of 0.1% FA in water while mobile phase B was composed of 0.1% FA in acetonitrile. Operating column temperature was 40ºC. Injection volume was 5 µL. The LC method details are summarized in Table 1.

Table 1. LC method.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0.0	*99.8*	*0.2*
2.0	*99.8*	*0.2*
5.0	*98*	*2*
11	*75*	*25*
13	*2*	*98*
17	*2*	*98*
18	*99.8*	*0.2*
20	*99.8*	*0.2*

Mass spectrometry: For each component, 2 MRM transitions were monitored with a few exceptions when only 1 MRM was available for analytes ionized in positive and negative mode. This allows for comparisons of ion ratios to help identify potential peak integration issues.

Scheduled MRM algorithm pro was used to optimize cycle times and maximize dwell times for each MRM transition (Figure 3). By scheduling transitions around the expected retention time of an analyte, the sMRM method enables monitoring of significantly more MRMs simultaneously without sacrificing superior analytical precision.

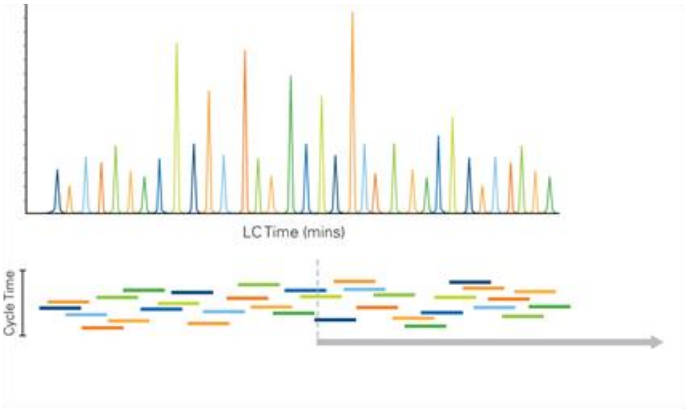

Figure 3. Scheduled MRM algorithm pro. Each MRM transition was monitored in each retention time window ensuring the simultaneous monitoring of several MRM transitions in a single LC run. This approach maintains the maximum dwell times and optimal cycle times for accurate quantification.

The final method contains 178 MRMs in positive and 54 MRMs in negative ionization mode which enables monitoring of over 110 media analytes over a 20 min run (Figure 2). Detailed MS parameters are shown in Table 2. Fast polarity switching allowed analysis in positive and negative ionization modes within 1 method.

Table 2. MS parameters.

Parameter	Value
Ion source gas 1	*40 psi*
Ion source gas 2	*70 psi*
Curtain gas	*40 psi*
CAD gas	*9*
Source temperature	*500°C*
Ion spray voltage	*1500 V/–1500 V*

Data processing: Scheduled MRM data was processed using the Analytics module in SCIEX OS software 2.0. T-test and PLS-DA were performed using MarkerView software.

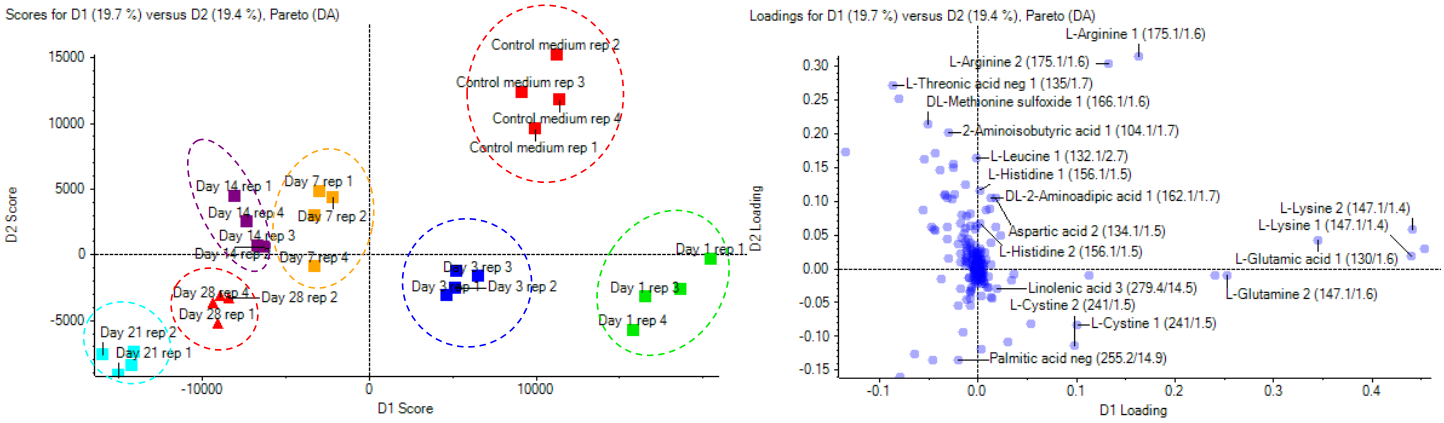

Figure 4. PLS-DA score and loading plots of cell culture media and spent media at different cell growth stages. Control media and media at day 1, 3, 5, 7, 14, 21 and 28 were analyzed. Each stage of cell growth was grouped separately from the control media with the earlier timepoints being closer together compared with later cell growth stages.

Workflow overview

The developed method was successfully applied for the analysis of secreted metabolites in the hASCs cell line. Components were monitored at day 1, 3, 7, 14, 21 and 28. Each sample was analyzed in 4 replicates. A control media was also analyzed separately to monitor components and changes from the initial stages of the experiment.

The fast polarity switching and the high sensitivity on the SCIEX 7500 system enabled monitoring of 196 transitions in positive ion mode and 76 transitions in negative ion mode within a single run. The most sensitive transition was applied for all the analyses. In the following results, representative examples were chosen to showcase the quantitative trend components and statistical comparisons using t-test.

Statistical analysis and quantitative trends at different stages of cell growth

PLS-DA was performed and loading plots were generated for the dataset. Figure 4 distinguishes groups by each growth day (1, 7, 14, 21 and 28 days) and grouped by initial growth days (1 and 3) which are located at highest positive values of D1 score (red and purple), middle growth days (7 and 14), at positive values of D2 score (green and blue) and end days (21 and 28) at highest negative values of both D1 and D2 (yellow and orange). There is a clear difference between the control cell culture media and the spent media from day 3 to 28.

Figure 5 shows the extracted ion chromatograms (XICs) at different cell growth stages for L-valine, glycerophosphocholine, succinic acid and lactic acid as representative examples. Here, L-valine and glycerophosphocholine were monitored in positive ion mode while succinic acid and lactic acid were analyzed in negative ion mode. As a result, the method can be applied to monitor components using the most sensitive polarity technique in a single analysis.

In Figure 6, representative compounds are shown to demonstrate trends observed over the course of cell growth. Example components were plotted using 4 replicates at each time point. Quantitative trends were processed using MarkerView software. Conclusions were drawn from t-tests to demonstrate statistical significance of the trend profile.

An overall summary of the t-test results for components which indicated a defined and statistically significant quantitative trends are shown in Table 3. Statistically significant quantitative trends were indicated by a p-value of less than 0.05. A total of 7 components were upregulated during the cell growth stages including DL-methionine sulfoxide, gluconic acid, glycerophosphocholine, lactic acid, L-asparagine, L-threonic acid and succinic acid.

Moreover, 6 components were downregulated during the stages of cell growth which involved L-arginine, L-glutamic acid, L-glutamine, L-lysine, nicotinic acid and pyruvic acid. The remaining metabolites had p-values greater than 0.05 indicating no significant quantitative trend over the course of growth.

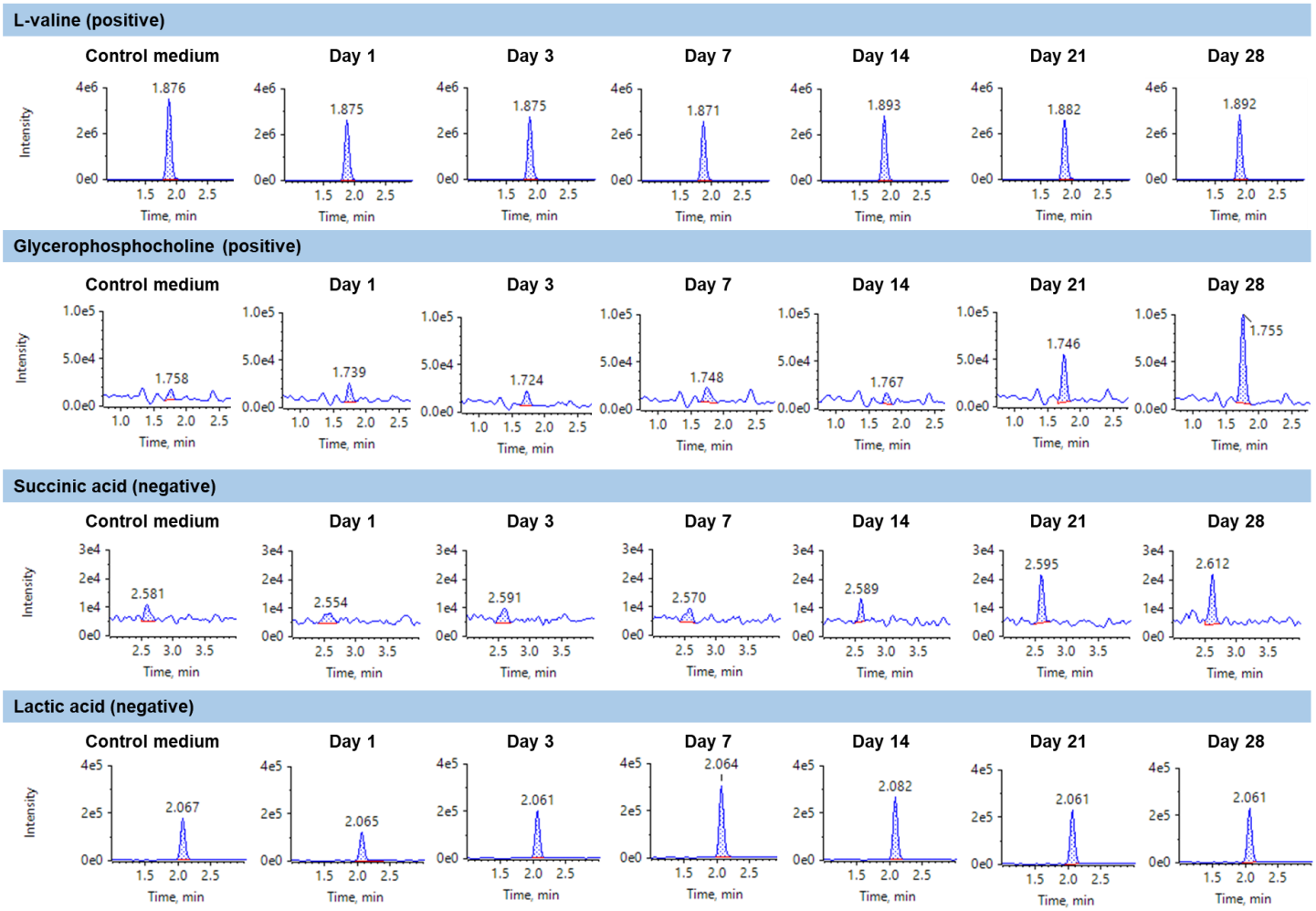

Figure 5. Monitoring control and spent media for representative components in positive and negative ion mode. Representative examples include L-valine, glycerophosphocholine, succinic acid and lactic acid. L-valine and glycerophosphocholine were measured in negative ion mode while succinic acid and lactic acid were analyzed in positive polarity. Control media and media at day 1, 3, 5, 7, 14, 21 and 28 were analyzed.

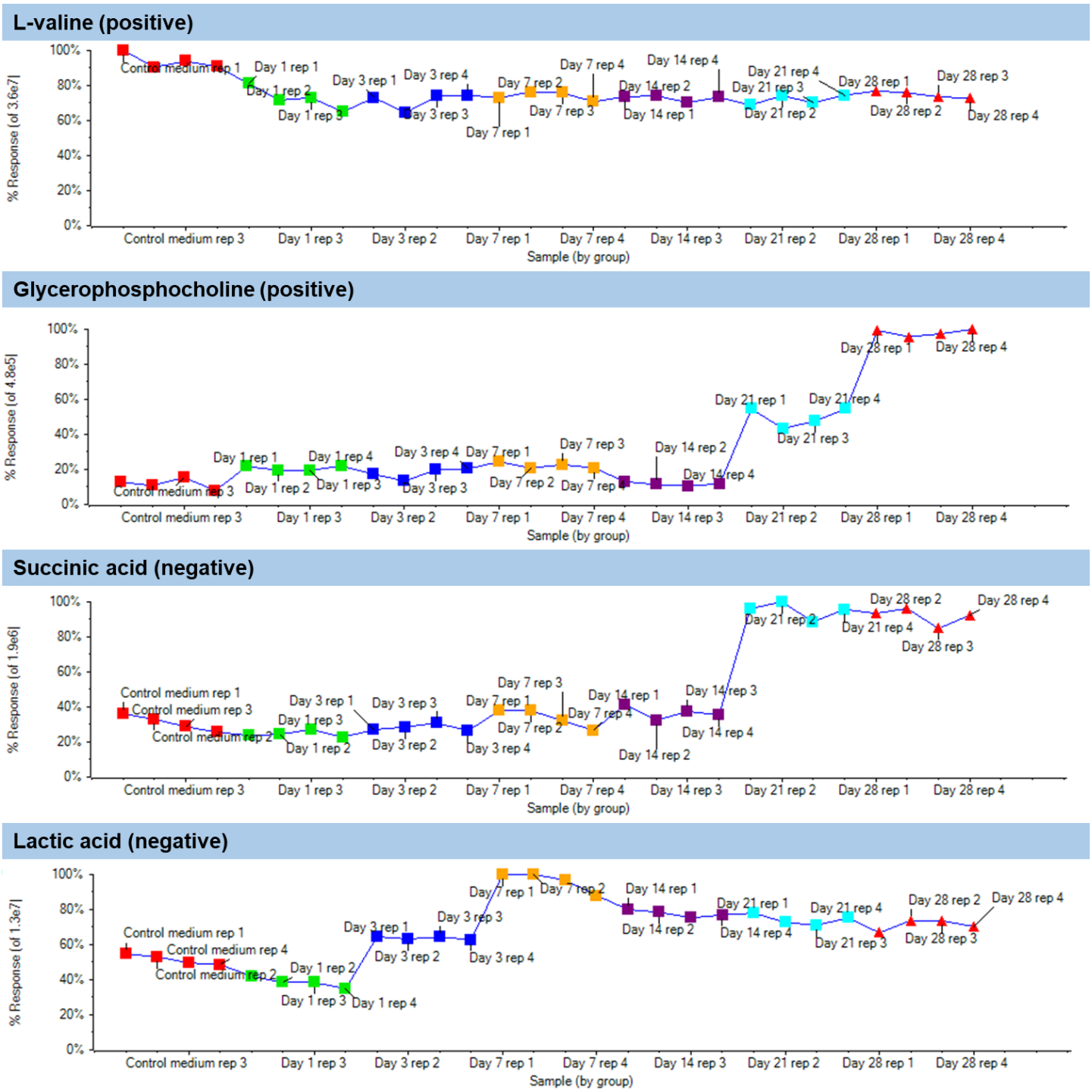

Figure 6. Quantitative trends of representative components in control and spent media at different cell growth stages. Representative examples include L-valine, glycerophosphocholine, succinic acid and lactic acid. Control media and media at day 1, 3, 5, 7, 14, 21 and 28 were analyzed in 4 replicates.

Table 3. T-test results.

Compound	p-value	Quantitative trend
DL-methionine sulfoxide	$1.10x10^{-4}$	*Upregulation*
*Gluconic acid**	$3.61x10^{-6}$	*Upregulation*
Glycerophosphocholine	$1.78x10^{-2}$	*Upregulation*
*Lactic acid**	$2.41x10^{-9}$	*Upregulation*
L-arginine	$2.80x10^{-4}$	*Downregulation*
L-asparagine	$4.74x10^{-5}$	*Upregulation*
L-glutamic acid	$2.22x10^{-3}$	*Downregulation*
L-glutamine	$5.46x10^{-8}$	*Downregulation*
L-lysine	$6.19x10^{-9}$	*Downregulation*
*L-threonic acid**	$3.51x10^{-6}$	*Upregulation*
Nicotinic acid	$5.41x10^{-3}$	*Downregulation*
Pyruvic acid	$6.13x10^{-6}$	*Downregulation*
*Succinic acid**	$4.60x10^{-4}$	*Upregulation*

**Monitored in negative ion mode.*

Studying the biological relevance of the observed trends

Statistically significant quantitative trends were examined for biological relevance over the course of cellular growth. Lactic acid, pyruvic acid and gluconic acid are involved in glycolysis and fermentation which are the main energy production pathways in 3D culture systems. In particular, lactic acid increases as the result of pyruvic acid reduction.[3] L-arginine, L-glutamic acid, L-glutamine and L-lysine decrease as the *in vitro* culture proceeds. These are proteinogenic amino-acids which get incorporated in proteins under biosynthetic conditions, such as in 3D culture and differentiation, given the demand for extracellular matrix (ECM) protein synthesis to maintain pellet stability and functionality.[4] Glycerophosphocholine is involved in cellular signaling pathways during cellular differentiation[5] and therefore, indicates an upregulation behavior.

As a result, through this analytical approach, the observed variation in metabolite levels were in agreement with the known biological activity.

Conclusions

- Sensitive monitoring of over 110 cell culture metabolites secreted from hASCs was achieved in under a 20 min run time
- A single platform method was applied with fast polarity switching for comprehensive analysis of metabolites in positive and negative modes using the SCIEX 7500 system
- Statistical analysis and quantitative trends were performed and generated using MarkerView software with capabilities of PLS-DA scoring and loading plots to demonstrate data correlation
- Statistically significant quantitative trends were in agreement with the biological activity over the course of cellular growth

References

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Headquarters
500 Old Connecticut Path | Framingham, MA 01701 USA
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