Quantitative and Qualitative Analysis of Cell Culture Medium Using SWATH® Acquisition

Featuring Data Independent Acquisition on SCIEX TripleTOF® 6600 System

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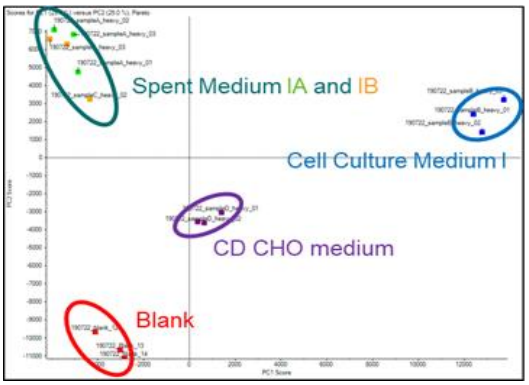
Biotherapeutic production requires precise monitoring of cell culture medium components across development and manufacturing stages. Cell culture media (CCM) and feeds tailored to specific cell lines maintain or improve the process yield, drug efficiency, safety and quality consistency. While there is a vital need to develop analytical methods for cell culture media (CCM) analysis, the diversity of compound classes and wide dynamic range of their natural abundances make the assay development challenging.

Recently, SCIEX introduced a MRM based workflow to quantify 110 cell culture components with a single LC-MS method.[1] This MRM workflow provides superior quantitation of the targeted CCM analyte in the defined list. Beyond the targeted quantitation workflow, there are also increased discussions in this area of research on simultaneously quantifying and confirming all analytes (expected or unexpected) in the CCM. In this aspect, SWATH® acquisition has been attracting significant interests, as it provides high resolution MS/MS level quantification and confirmation/identification of every detectable compound. Herein, a data independent workflow using SWATH® acquisition is demonstrated for comprehensive CCM analysis.



Statistical Comparison Between Different Media. PCA plot shows a good separation between blank, cell culture media and spent media.

ExionLC™ System coupled to TripleTOF® 6600 LC-MS/MS system and SCIEX OS 1.5 for data processing

Key Features of Cell Culture Media Analysis using SWATH® Acquisition

- SWATH® acquisition on the TripleTOF® system offers:
  - Wide analyte coverage by quantifying and confirming every ESI MS detectable compound in the medium
  - Superior quantitation quality based on high resolution MS/MS spectra, compared to single MS spectra
  - Ease of method development by using generic method parameters with minimum method optimization requirement
  - Variable Q1 window acquisition by optimizing window width based on *m/z* density of precursors, allows for greater selectivity in chromatographic periods of high precursor ion density
- Phenomenex Kinetex® F5 column provides excellent resolution of target analytes across different chemistries
- The Accurate Mass Metabolite Spectra Library allows compound confirmation with over 550 culture media components and metabolites for biological processes
- Powerful, comprehensive software solution including SCIEX OS-Q and MarkerView™ software offers versatile qualitative and quantitative workflows with statistical analysis

Table 2. Summary of mass spectrometry parameters.

Parameter	Value	Parameter	Value
MS mass range	*50-700 m/z*	*MS/MS mass range*	25-700 *m/z*
MS accumulation time	*100 ms*	*MS/MS accumulation time*	*35* ms
Curtain gas:	*30 psi*	*Source temperature:*	400 *°C*
Ion source gas 1:	*50 psi*	*Ion source gas 2:*	50 *psi*
Polarity:	*+ or -*	*Ion spray voltage:*	5500 or -4500V

Table 1. The summary of cell culture media component coverage among various compound groups.

Component group	Number of components
Amino acids	*39*
Vitamins	*15*
Carbohydrates	*4*
Fatty acids	*5*
Nucleic acids	*17*
Others	*32*

Methods

Sample preparation: The stock solutions of individual standards (1 mg/ml) from various compound classes (Table 1) were prepared with different solvents depending on compound solubility. The final master mix was prepared by mixing the stock solutions, resulting in final concentration range from 6.67 to 20 µg/ml. CD CHO medium (Gibco), cell culture medium I and its spent media were diluted 5-fold with 0.1% formic acid in 50% acetonitrile, and centrifuged. The supernatants were collected and further diluted 60 fold with 0.1% formic acid prior to LC-MS analysis.

Chromatography: Samples were subjected to LC-MS analysis in triplicate by using a TripleTOF® 6600 LC-MS/MS system coupled with an ExionLC™ system. Analytes were separated using a Phenomenex Kinetex® F5 column (150 mm x 2.1 mm ID, particle size 2.6 µm). The LC run time was 20 min at a flow rate of 200 µL/min. Mobile phase A was 0.1% formic acid in water, and mobile phase B was 0.1% formic acid in acetonitrile. Column oven temperature was 40°C and the injection volume was 5 µL.

Mass Spectrometry: The data were generated with variable window SWATH® acquisition using 20 variable windows. The window widths were determined based on the MS ion intensity distribution using the SWATH® acquisition Variable Window Calculator.[2] The method details are listed in table 2.

Data processing: The data were analyzed using SCIEX OS 1.5 software with details listed below.

In the targeted workflow (quantitation and confirmation), an MRM transition library[1] with 196 positive and 71 negative entries (Table 3) was incorporated into SCIEX OS to generate the quantitation method. Peak integration was performed with MQ4 algorithm. For compound confirmation, the acquired MS/MS spectra were matched against MS/MS spectra in the built-in high resolution spectral library. This built-in spectral library contains entries from the SCIEX Accurate Metabolite Spectral library, Antibiotics and NIST library.

In the untargeted compound screening workflow, the LC-MS peak picking option was set as semi-exhaustive. All LC-MS peaks for the same analyte in the forms of different charge states or adducts were grouped together and analyzed as a single entry, which significantly simplified the data process. To achieve the optimal performance of compound identification, three processing functions were used: 1) FormulaFinder: identify possible compound formula based on TOF MS spectra (compound molecular weight); 2) ChemSpider: link compound formula with in silico MS/MS database predicted from compound structures; 3) library search: search MS/MS spectra with built-in spectral library.

Statistical analysis: MarkerView™ software was used for analyzing cell culture media and spent media samples with statistical tools, including principle components analysis (PCA), t-test, volcano plots and differential abundance visualization between media.

Overview of SWATH® acquisition

In this project, SWATH® acquisition was utilized to develop a comprehensive workflow for quantitative compound profiling in the cell culture medium. SWATH® acquisition, also known as data independent acquisition (DIA), collects MS and MS/MS information from all ionized compounds, with no requirement to obtain sample information prior to data generation. Compared to the data dependent acquisition (DDA), SWATH® acquisition workflow demonstrates significant advantages in multiple aspects: 1) analyte coverage: it allows to keep permanent quantifiable digital records of every detectable compound in the medium; 2) quantitation capability: it provides high resolution MS/MS based quantitation; 3) ease of method development: it uses generic method parameters with minimum method optimization requirement.

The SWATH® acquisition workflow is demonstrated in Figure 1. The Q1 quadrupole transmits ions within a wide mass range (m/z isolation window) through to the collision cell. This produces a MS/MS spectrum which is a composite of all the analytes within this wide Q1 m/z window. Because the fragment ions are high resolution, high quality XICs can be generated post-acquisition to produce the MRM-like data. This Q1 window can be stepped across the mass range, collecting full scan composite MS/MS spectra at each step, with an LC compatible cycle time.

SWATH® acquisition also offers variable window acquisition strategy[3] by allowing the user to vary the size of the Q1 isolation window based on the density of analyte precursor masses (Figure 2). In this project, an optimal method with 20 variable isolation windows was built to allow full mass range coverage and high quantitation performance

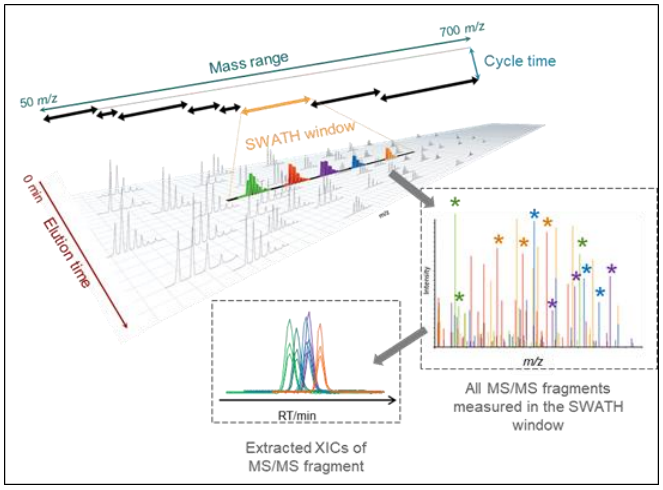


Figure 1. The general workflow of SWATH® acquisition.

Cell culture media analysis workflow using SWATH® acquisition

The above described SWATH® acquisition workflow was applied for both qualitative and quantitative analysis of cell culture medium (Figure 3). In the targeted (quantitation and confirmation) workflow, an MRM transition library[1] was incorporated into SCIEX OS to generate quantitation method. Peak integration was performed with the autopeak algorithm. The abundant fragment ions of the target analyte were automatically extracted by software for peak integration. Figure 4 showed an example of choline quantification and confirmation in master mix, in which the calibration curves (Figure 4A) and XICs (Figure 4B) of multiple fragment ions, TOF MS spectrum (Figure 4C) and MS/MS library matching (Figure 4D) were presented. Figure 5 demonstrated the quantification of selected analytes in serial dilution samples with positive or negative ionization. Another example (Figure 6) was shown as the quantification of

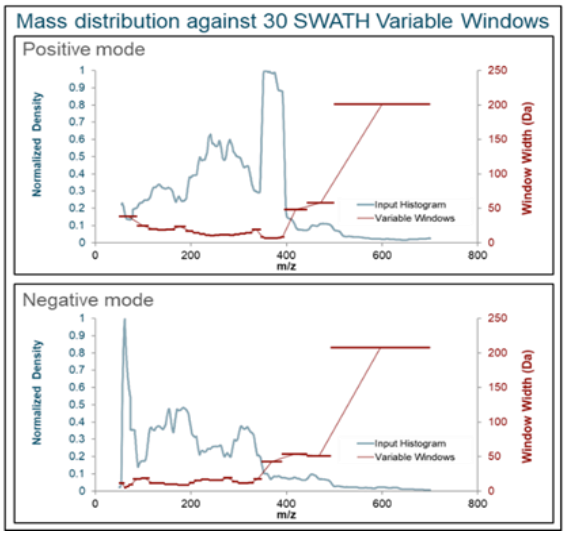


Figure 2. Graphic representation of windows size variation. The size of the Q1 isolation window (red line) is determined by the density of component precursor masses (blue line).

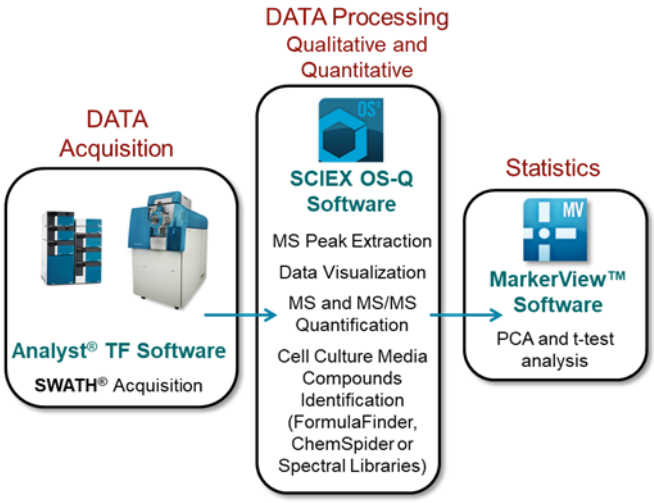


Figure 3. Cell Culture media workflow: from data acquisition to trends visualization. Data are acquired in SWATH® acquisition mode, qualitatively and quantitatively processed with SCIEX OS-Q, and statistically analyzed with MarkerView™.

multiple components in the Gibco's CD CHO cell medium, in which the analyte abundances covered over four orders of magnitudes.

In the untargeted compound screening workflow, both MS and MS/MS spectra were processed by SCIEX OS-Q for compound identification (Figure 6). The TOF MS spectrum was linked with predicted compound formula by FormulaFinder and searched against ChemSpider for structure matching and fragment ion prediction. The MS/MS spectrum is searched against MS/MS spectral library wherein multiple libraries (the SCIEX accurate

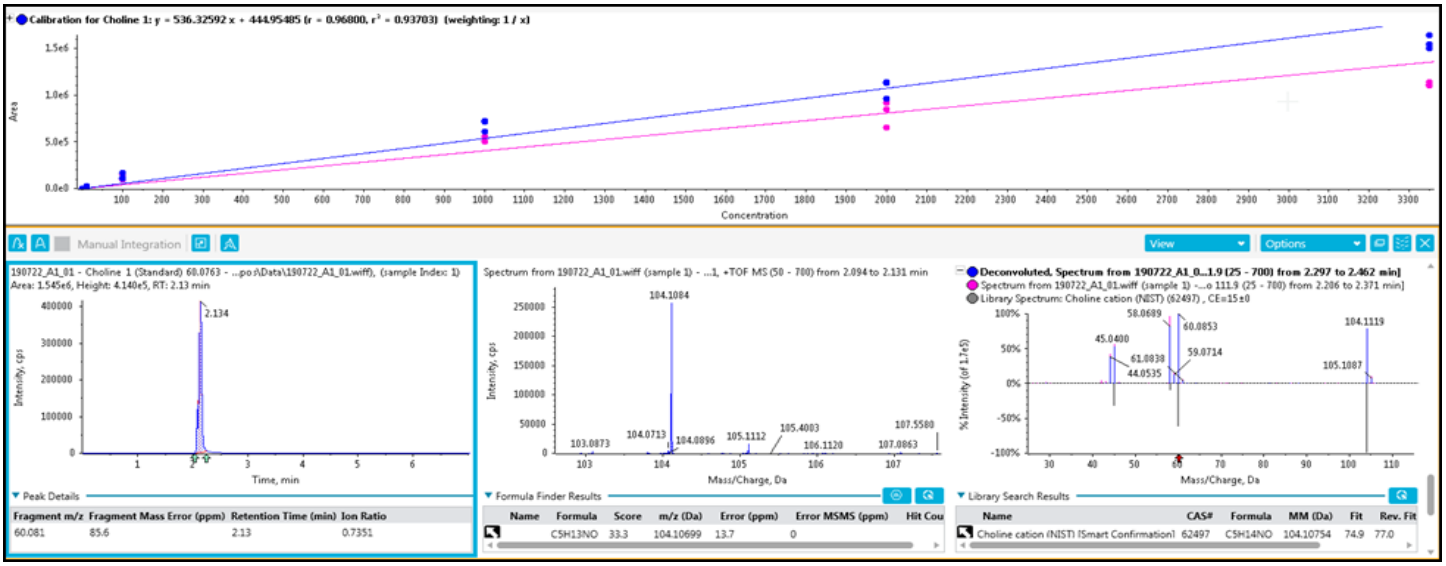


Figure 4. Choline quantification and confirmation in master mix. A) calibration curves; B) (left) XICs of multiple fragment ions; C) (middle)TOF MS spectrum with FormulaFinder matching; D) (right) MS/MS spectrum with library matching.

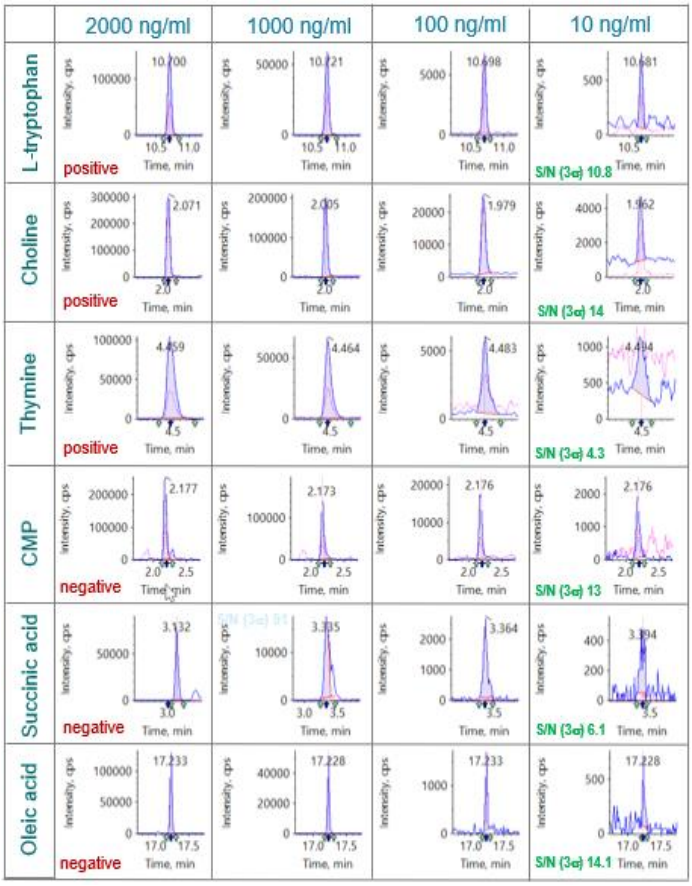


Figure 5. XICs of representative compounds in serial dilution master mix (10, 100, 1000 and 200 ng/mL) with positive or negative ionization.

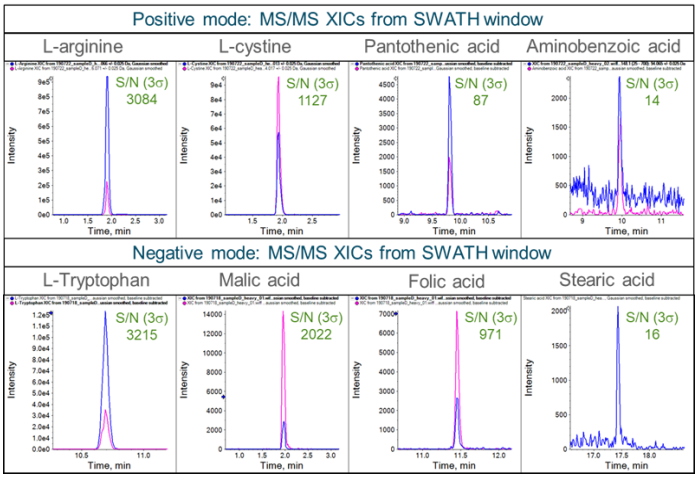


Figure 6. XICs of representative compounds from Gibco's CD CHO medium. Selected analytes abundances covered four orders of magnitudes. The medium sample was diluted 300 fold prior to analysis.

spectra metabolite and antibiotics library plus a subset of the NIST spectral library) could be combined to achieve optimal compound coverage. This workflow significantly reduced false discovery rate by providing MS/MS level confirmation in addition to the retention time and precursor mass information. Figure 7 shows an example of unknown compound identification in the medium sample. The compound was identified as Spermine which is used in serum free cell culture medium to improve expression levels recombinant protein, cell growth and viability.[4]

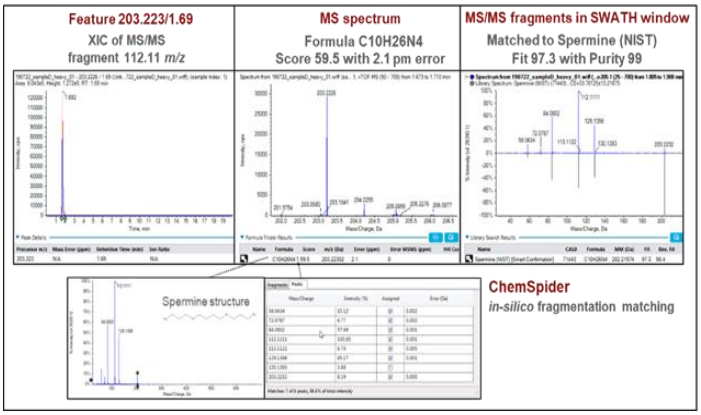


Figure 7. Untargeted compound screening using SCIEX OS-Q software. Spermine was identified as an addition to known cell culture medium compound. The TOF MS spectrum ([M+H]+; 203.2226 m/z) was matched to compound formula (C10H26N4) and searched against ChemSpider. The MS/MS spectrum was searched against MS/MS spectral library, showing a good matching (Fit 98.3, Purity 99%) between measured MS/MS spectrum (blue MS/MS spectrum) and library spectrum (grey MS/MS spectrum).

Statistical Analysis

SCIEX MarkerView™ software was applied in cell culture media analysis workflow for statistical evaluation. The data generated from different cell culture media and spent media samples were analyzed in SCIEX OS-Q and imported to MarkerView™ software for principal components analysis and analyte abundance visualization among multiple samples. As shown in Figure 8A, two spend media were grouped together and well separated from the "fresh" cell culture medium (cell culture medium I.). This suggested that there were significant variations in component regulation between start and end point of biotherapeutics production. As shown in Figure 8B, levels of L-serine varied among media samples (decreased during production). L-Serine (a non-essential amino acid) stimulates cell growth and prolong cell viability.[5] The decrease of its level could be related to its involvement in the nucleic acid precursor metabolism to support cell proliferation, through the folate (THF) cycle and the methionine cycle.[5]

Conclusions

A powerful data independent workflow using SWATH® acquisition was demonstrated for comprehensive cell culture media analysis. It provided 1) the high resolution MS/MS based quantitation and confirmation of every detectable compound in media, 2) the full data processing software solution including library search and statistical analysis. Combined with the previously reported MRM workflow,[1] a versatile application solution for cell culture media analysis was offered serving different needs during biotherapeutics manufacturing.

References

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2. SWATH® acquisition Variable Window Calculator - Excel tool. Download from http://sciex.com/support/software-downloads.

3. Improved Data Quality Using Variable Q1 Window Widths in SWATH® Acquisition, SCIEX Technical Note RUO-MKT-02-2879-B.

4. Method for culturing mammalian cells to improve recombinant protein production, Patent US20100221823

5. One-Carbon Metabolism in Health and Disease, Cell Metabolism, 25, 1, 27-42, 2017

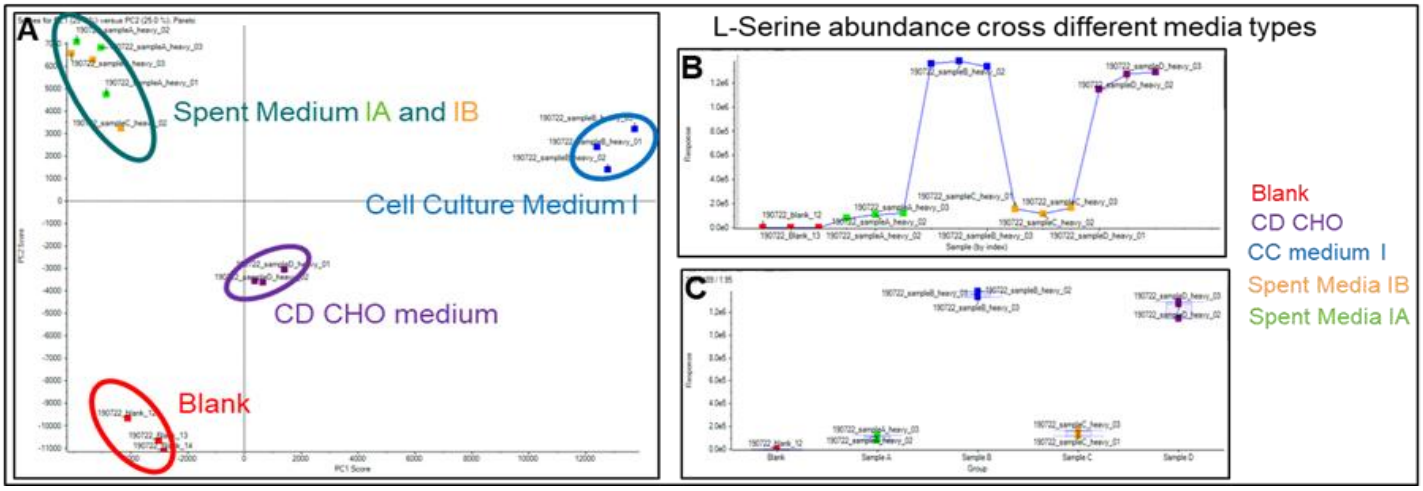

Figure 8: Statistical comparison between different media and L serine uptake during biotherapeutic production. A) PCA plot shows a good separation between different media, B) quantitative trend of L-serine between different samples and C) Box plots of L-serine in different samples. Media are color coded listed on the left side of figure.

Table 3. List of components in the MRM library for the targeted workflow.

Cell Culture compound	Group
L-Alanine	Amino acid
Beta-Alanine	Amino acid
L-Arginine	Amino acid
L-Aspartic Acid	Amino acid
L-Cystine	Amino acid
L-Glutamic acid	Amino acid
Glycine	Amino acid
L-Histidine	Amino acid
L-Isoleucine	Amino acid
L-Leucine	Amino acid
L-Lysine	Amino acid
L-Methionine	Amino acid
L-Phenylalanine	Amino acid
L-Proline	Amino acid
L-Serine	Amino acid
L-Threonine	Amino acid
L-Tyrosine	Amino acid
L-Valine	Amino acid
L-Tryptophan	Amino acid
γ-Amino-n-butyric acid	Amino acid
L-alpha-Amino-n-butyric acid	Amino acid
DL-β-Aminoisobutryic acid	Amino acid
L-Carnosine	Amino acid
L-Citrulline	Amino acid
L-Cystathionine	Amino acid
Ethanolamine	Amino acid
L-Homocystine	Amino acid
δ-Hydroxylysine	Amino acid
Hydroxy-L-proline	Amino acid
1-Methyl-L-histidine	Amino acid
3-Methyl-L-histidine	Amino acid
L-Ornithine	Amino acid
L-Sarcosine	Amino acid
L-Asparagine	Amino acid
L-Glutamine	Amino acid
L-Methionine sulfoxide	Amino acid
L-pyroGlutamic acid	Amino acid
N-Acetyl -L-aspartic acid	Amino acid
N-Acetyl-L-cysteine	Amino acid
trans-4-hydroxy-L-Proline	Amino acid
L-Norvaline	Amino acid
sarcosine	Amino acid
L-Kynurenine	Amino acid
linolenic acid	Fatty acid
linoleic acid	Fatty acid
oleic acid	Fatty acid
stearic acid	Fatty acid
palimitic acid	Fatty acid
Adenine	Nucleobase
Guanine	Nucleobase
thymine	Nucleobase
Uracil	Nucleobase
hypoxanthine	Nucleobase
Xanthine	Nucleobase
2'-Deoxycytidine	Nucleoside
Adenosine free base	Nucleoside
Adenosine 5'-monophosphate	Nucleoside
Cytidine	Nucleoside
Cytidine 5'-monophosphate	Nucleoside
Guanosine	Nucleoside
Guanosine 5'-monophosphate disodium hydrate	Nucleoside
Inosine	Nucleoside
Thymidine	Nucleoside
Uridine	Nucleoside
Xanthosine dihydrate	Nucleoside
Sucrose	Sacharides
D-(+)-glucose	Sacharides
D-(+)-glucosamine hydrochloride	Sacharides
(-)-Tocopherol acetate	Vitamin
Biotin	Vitamin
Cyanocobalamin	Vitamin
D-Pantothenic acid hemicalcium	Vitamin
Folic acid	Vitamin
L-Ascorbic acid	Vitamin
L-Ascorbic acid 2-phosphate sesquimagnesium salt	Vitamin
Niacinamide	Vitamin
Nicotinic acid (niacin)	Vitamin
Pyridoxal hydrochloride	Vitamin
(-)-Riboflavin	Vitamin
ergocalciferol	Vitamin
sodium ascorbate	Vitamin
pyridoxine	Vitamin
(-)-alpha-Lipoic acid	Vitamin
Taurine	Others
2-isopropyl Malic acid	Others
2-oxovaleric acid	Others
Citric acid	Others
DL-A-Keto-B-methyl-n-valeric acid sodium	Others
DL-Isocitric acid trisodium hydrate	Others
DL-P-Hydroxyphenyllactic acid	Others
Fumaric acid	Others
Lactic acid	Others
Pyruvic acid	Others
Succinic acid	Others
malic acid	Others
D-gluconic acid sodium	Others
L-Anserine	Others
ALA-GLN	Others
gly-gln monohydrate	Others
4-Aminobenzoic acid	Others
Choline chloride	Others
L-2-Aminoadipic acid	Others
L-Pipecolic acid	Others
Uric acid	Others
Folinic acid calcium salt hydrate	Others
Penicillin G sodium	Others
2-Aminoethanol (monoethanolamine)	Others
Ethylenediamine	Others
Histamine free base	Others
O-Phosphoethanolamine	Others
Putrescine	Others
Phosphocholine chloride calcium salt tetrahydrate	Others
Glutathione oxidized	Others
L-glycerophosphocholine	Others
D-Threonic acid lithiumsalt	Others

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