Quantitative LC-MS Solution for Targeted Analysis of Cell Culture Media

Featuring the SCIEX QTRAP® 6500+ LC-MS/MS System

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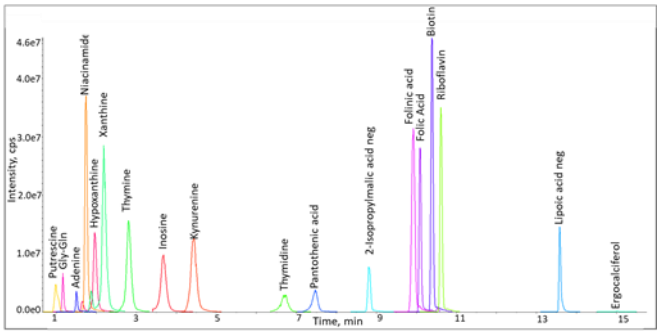
Introduction

Cell culture media (CCM) optimization is a critical step during the development and scale up of biotherapeutic production. In particular, the emphasis on quality by design (QbD) has made it necessary to understand how the components of CCM change during production and how these changes relate to product quality. There is a vital need to develop analytical assays that can provide comprehensive and accurate cell culture media profiling for a wide range of biotherapeutics types produced from or are themselves living cells.

Compared to traditional analytical and biosensor techniques, (*e.g.*, UV-visible spectrophotometry, nuclear magnetic resonance and Raman spectroscopy; liquid chromatography mass spectrometry (LC-MS) techniques provide a strong solution require for cell culture media analysis. High sensitivity, selectivity, speed, and robustness; enables unambiguous identification and quantification of a large number of analytes in a single analysis.

To enable rapid analysis of a wide array of cell culture media components, SCIEX has developed a CCM method on the QTRAP® 6500+ system coupled to the ExionLC™ system. This new CCM method targets important nutrients, including amino acids, vitamins, carbohydrates, fatty acids, nucleic acid, inorganic acids, and other essential compounds found in media. By offering sensitive, reproducible and robust quantification 110 key cell culture media components can be analyzed in a single LC-MS/MS method.



Representative Extracted Ion Chromatograms of selected components from compound library analyzed using a schedule MRM™ method

SCIEX QTRAP® 6500+ coupled to ExionLC™ System

Key Features of Cell Culture Media Method

- SCIEX QTRAP® 6500+ coupled to ExionLC™ system offers speed, high sensitivity, reproducibility and linear ion trap functionality
- Phenomenex Kinetex® F5 column provides excellent resolution of target analytes a cross different chemistries
- The MRM library identifies 110 key cell culture nutrients and contains two MRMs per compound with a few exceptions
- The Accurate Mass Metabolite Spectra Library for compound confirmation with over 550 essential metabolites for biological processes
- Powerful, comprehensive software solution - SCIEX OS-Q software offers versatile qualitative and quantitative workflows

Experimental Section

***Sample preparation*:** A cell culture master mix was prepared using standards from various groups is listed in Table 1 and contained the individual components listed in Table 2. A stock solution of individual standards (1 mg/ml) was prepared with different solvent depending on compound solubility. A final master mix was prepared containing all standards (6.67-20 µg/ml depending on analyte signal).

Cell culture medium (CD CHO medium, Gibco) was diluted 5-fold with 0.1% formic acid in 50% acetonitrile, and centrifuged. the supernatant was further diluted 60-fold with 0.1% formic acid.

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*

Chromatography: Analytes were separated on an ExionLC™ System using a Phenomenex Kinetex® F5 column (150 mm x 2.1 mm ID, particle size 2.6 µm). Total method time was 20 min at a flowrate of 200 µl/min. Mobile phase A was 0.1% formic acid in water, and mobile phase B was acetonitrile with 0.1% formic acid. Column oven temperature was 40°C. Injection volume was 5 µL.

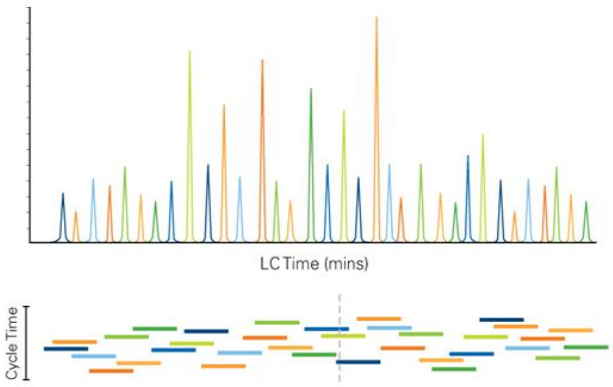


Figure 1. Scheduled MRM™ Algorithm Pro. Using knowledge of the elution time of each analyte, each MRM transition is monitored only during a short retention time window. This allows many more MRM transitions to be monitored in a single LC run, while still maintaining maximized dwell times and optimized cycle times.

Mass Spec: MRM parameters for 110 cell culture components were optimized by chemical standards for a SCIEX QTRAP® 6500+ and a Triple Quad™ 6500+ system. The scheduled MRM™ Algorithm Pro(sMRM) was used to optimize cycle times and maximized dwell times for each MRM transition. By scheduling transitions around expected retention time of an analyte, the sMRMs method allows for significantly more MRM transitions to be monitored simultaneously without sacrificing the superior analytical precision (Figure 1). Two MRM transitions were monitored for each analyte, with a few exceptions when only one MRM was available or analytes ionized in positive and negative mode. This allows for comparisons to standard ratios to help identify peak integration issues. This method contains 178 positive MRMs and 54 negative MRMs.

The MIDAS™ workflow was used to confirm components of the master mix based on their full MS/MS fragmentation pattern of each analyte.

Mass spectrometer settings were: curtain gas 30 psi, GS1 50 psi, GS2 50 psi, ISVF 4500 V or -4500 V, TEM. 400°C. Fast polarity switching allowed analysis in positive and negative ionization modes within one method.

Data processing: *Scheduled* MRM data was processed by using SCIEX OS-Q 1.5 software with a targeted quantitative workflow. The MQ4 algorithm was used for integration of analyte peaks. Confirmation of components was carried out using full MS/MS fragmentation pattern (MIDAS™) in SCIEX OS-Q 1.5 software with qualitative workflow and the Accurate Mass Metabolite Spectral Library (AMMSL).

Results and Discussion

One major challenge encountered in cell culture media analysis is the chromatographic separation and retention of various groups of components including isomers and polar analytes. Recently the use of HILIC has emerged as an approach for separation of some classes of CCM components. As the diversity of the components increases the advantage of HILIC decreases when compared to traditional reverse phase approaches. In addition the overall run times using HILIC are frequently longer due to extended re-equilibration and as such the retention time reproducibility may suffer.

SCIEX developed a CCM method that separates various classes of compounds (Table1) using the reversed phase chromatography. For this purpose we selected the Kinetex® F5 column. This chemistry provides separation with high resolving power for chemically different analytes based on five different interactions (hydrophobic, aromatic, electrostatic, steric/planar, and hydrogen bonding) which enables efficient separation over a

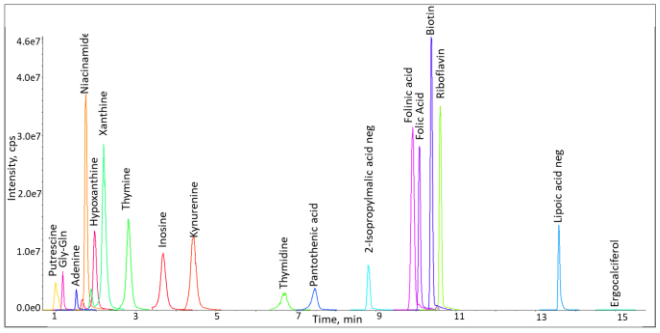


Figure 2. Representative Extracted Ion Chromatograms of selected components from component MRM library. Panel A shows various XICs of components from standard cell culture mixture.

wide range of molecular properties. Figure 2A illustrates extracted ion chromatograms of representative components from

cell culture media standard master mix, for clarity only one MRM per component for selected components are shown. As a proof of concept, the Gibco cell culture (CD CHO) medium was analyzed using this CCM method. As shown in Figures 3, a number of components are found in the CD CHO medium that align with the standard master mix used for method development. Due to concentration differences low abundance components are shown in the top panel of Figure 3 while the amino acids are presented separately in the bottom panel.

A particular challenge with generic methods for CCM analysis is the separation of closely related compounds. One example is the ability to distinguish between L-cysteine and L-cystine due to similar fragmentation patterns. L-cystine is one of five amino acids (arginine, cystine, glutamine, histidine, tyrosine) that are essential for the survival and growth of cells in culture[1]. L-cystine, the dimer of L-cysteine, is formed non-enzymatically through reversible oxidation of the thiol residue. Monitoring the ways that cells use these components may be important during a

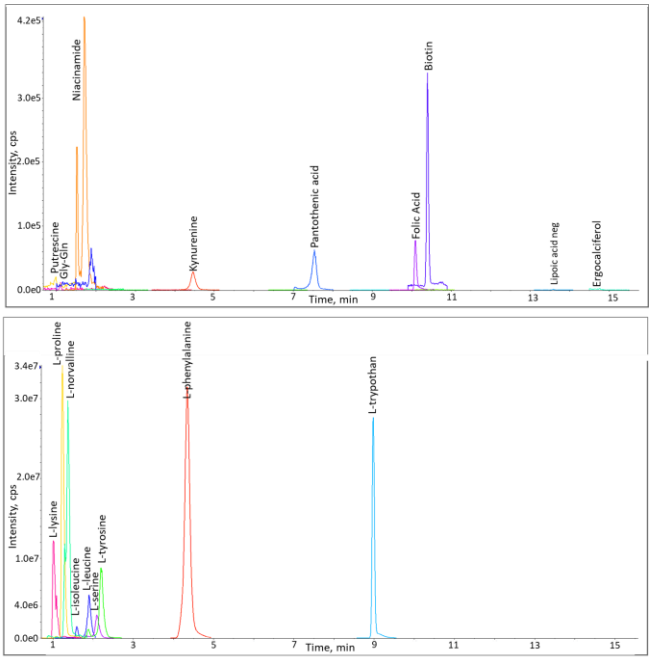


Figure 3. Top panel shows various XICs of low abundant components from Gibco's cell culture media (CD CHO medium). Bottom panel shows amino acids from Gibco's cell culture media (CD CHO medium).

development of successful medium. Using the method presented here, L-cystine and L-cysteine are well separated from each other, and also distinguished by unique MRMs (Figure 4A). Another example is detection of L-Arginine and its metabolites, such as L-ornithine and L-citrulline. L-citrulline converts to L-arginine[2] during biosynthesis. The separation of these components is shown in Figure 4B using MRMs for each.

Separation of isomers is a good indicator of sufficient column selectivity. Figure 5, top panel, shows a good baseline separation of L-leucine and L-isoleucine. Baseline separation and unique MRMs enable correct assignment of each isomer.

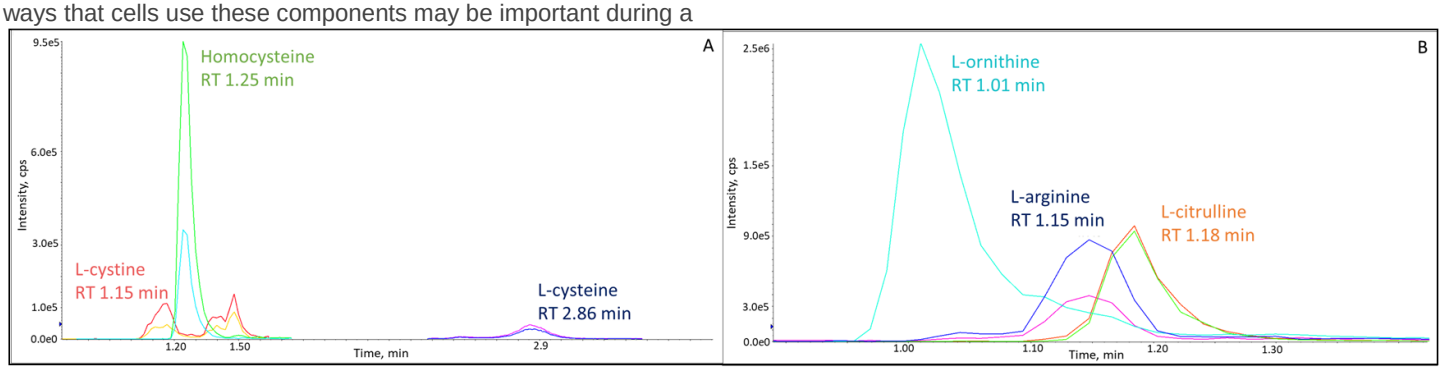


Figure 4. Example of separation between amino acid and its homologs, its metabolite and dimer. Panel A shows extracted ion chromatograms of Cysteine, Cystine and Homocysteine MRMs. Panel B shows extracted ion chromatograms of L-Arginine, L-Ornithine and L-Citrulline MRMs. These examples were extracted from separation of cell culture standard master mix on Kinetex® F5 column within 20 min.

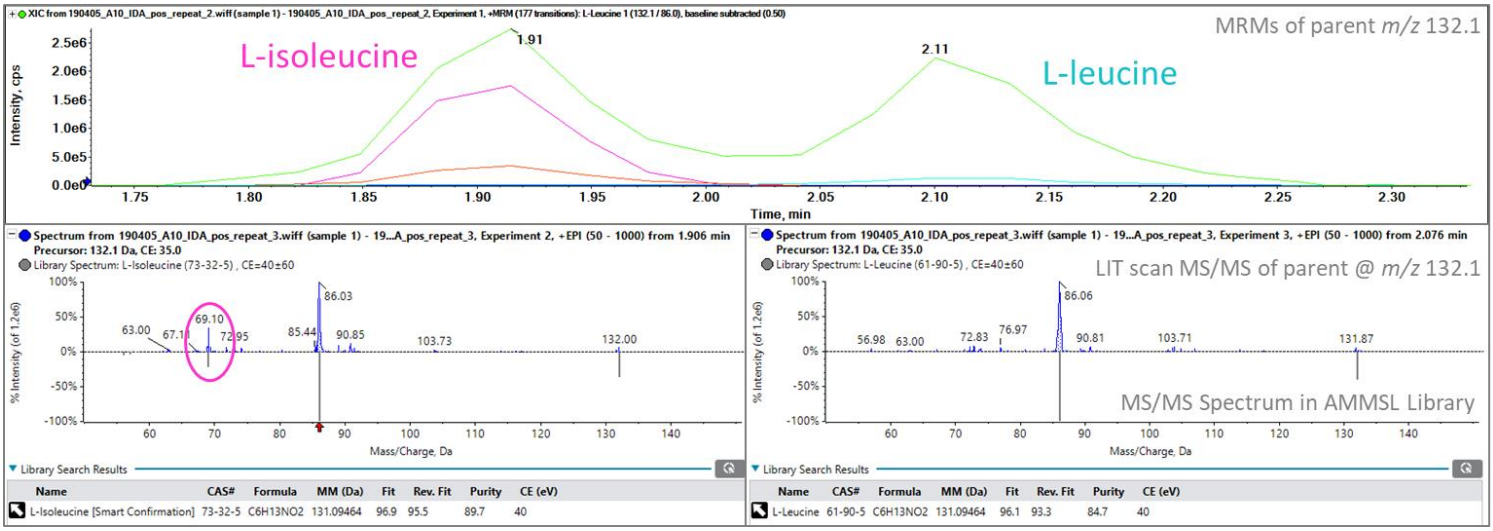


Figure 5. Representative MIDAS™ workflow for L-Leucine and L-Isoleucine with compound match from the Sciex Accurate Metabolite Spectra (AMMSL) Library. Top panel illustrates separation of L-Leucine isoforms with XICs from corresponding MRMs. Bottom panels shows in the blue spectrum the LIT scan of all measured MS/MS ions from L-Leucine and L-Isoleucine parent ion and the grey spectrum shows the library spectrum of L-Isoleucine and L-Leucine from the AMMSL Library.

In cases where assignment of the correct component, or identification of a new component, is required the MIDAS™ workflow enables collection of MRM and IDA data within the same acquisition. Figure 5 bottom illustrates confirmation of L-leucine and L-isoleucine using data acquired using the MIDAS™ workflow. As shown, the identification of each isomer is based on MS/MS spectral matching between the experimental and theoretical MS/MS spectra from the AMMSL library. In this example, the isomer position is confirmed by a specific L-isoleucine MS/MS fragment (69.10 *m/z*) which is not present in the L-leucine MS/MS pattern. For each component the match score was above 90 providing high confidence in assignment and therefore its use in quantification.

Quantitation of components from cell culture medium can be challenging and analyte dependent, especially when substantial concentration differences and multiple components are simultaneously analyzed. The lower limit of quantification (LLOQ) and linear dynamic range (LDR) was investigating during the method evaluation. In Figure 6, calibration curves are representing linear respond of selected component per group. As an example, L-tryptophan has linear range from 0.001 ng/mL to 3350 ng/mL and LLOQ of 0.025 ng/ml with signal to noise (S/N) of 46. On average LDRs across all of the components in the method range from 2.5 to 6 orders of magnitude.

To monitor the levels of cell culture media components during the different stages of biotherapeutics production, SCIEX OS software offers a visual way to gauge the level of nutrition available for cell growth through its integrated plot functionality (concentration metric plot), the graphical representation of results across different stages is shown in Figure 7.

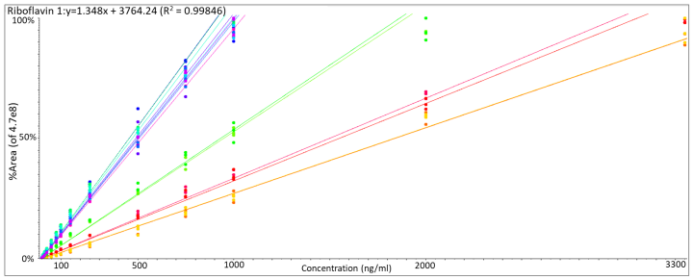


Figure 6. Linear calibration curves of representative cell culture component per group measured in positive and negative mode. Concentration slope for T-Tryptophan and Linoleic acid is from 0.001 ng/mL to 3350 ng/mL (orange and red lines), Adenosine is from 0.001 ng/mL to 2000 ng/mL(green lines), D-threonic acid and Riboflavin pos/neg is from 0.001 ng/mL to 1000 ng/mL, and 2-Isopropylmalic acid 0.05 ng/mL to 2000 ng/mL(blue, purple and turquoise lines). Outliers were removed and average R^2 value this components is 0.98893. Y-axis is presented as %Area of 4.7e8 and X-axis is concentration in ng/ml.

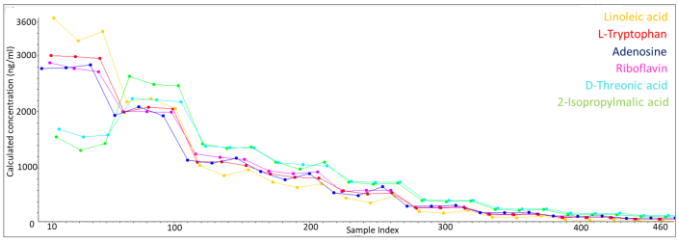


Figure 7. Metric plots reflect concentration variation between samples. Cell culture components: - linoleic acid (yellow), L-tryptophan (red), adenosine (blue), riboflavin (pink), D-threonic acid (light blue) 2-isopropylmalic acid (green). Component quantity is shown from the highest to lowest calibration point normalized to highest concentration.

Figure 8. Extracted Ion Chromatograms shows two MRMs per selected compound from cell culture group class. In the cell culture standard mixture the LDR vary from 2- to 6-orders of magnitude depending. Blue XIC is MRM and pink XIC is MRM2 for selected compound. The Signal to Noise ratio values indicating the LLOQ of compound. * 2x higher load on a column.

This visualization tool compares the different stages of cell culture media cultivation for successful large scale biotherapeutics production. The measured quantity from the highest to lowest calibration point is normalized to highest concentration and plotted against time (Figure 7). In the tool, representative components for each cell culture media component group are represented by different colors: amino acids – L-tryptophan (red), vitamins – riboflavin (pink), carbohydrates- D-threonic acid (light blue), fatty acids – linoleic acid (yellow), nucleic acids – adenosine (blue line) and organic acids – 2-isopropylmalic acid (green line). Initially, the plot shows lower concertation of carbohydrate (D-threonic acid) and organic acid (2-isopropylmalic acid) at the highest point of calibration, this most probably results of detector saturation (Figure 7).

Conclusions

Herein, a quantitative LC-MS solution for targeted cell culture media analysis was presented. This solution is based on the use of a SCIEX QTRAP® 6500+ mass spectrometer coupled to ExionLC™ system, and was designed specifically to meet industry demands and overcome technical challenges for assay development. This cell culture media analysis method offers:

- Superior separation of critical cell culture media components over a broad range of chemistries
- Measurement of polar and nonpolar as well as positive and negative polarity components within a single method
- An MRM-driven method that is easily convertible to other chromatographic systems
- High sensitivity and dynamic range when using a SCIEX QTRAP® 6500+ and Triple Quad™ 6500+ system
- A full software solution for direct quantitation and principal components based graphical visualization
- Enhanced robustness due to the Iondrive™ Turbo V source that delivers high sensitivity for both positive and negative analysis
- A heavy internal standard kit that will allow for accurate quantitation of individual component groups within cell culture media (forthcoming)

References

1 Eagle, H. (1959) Science, 130, pp. 432.

2 Schinke, R. (1964) The Journal of Biological Chemistry, 239, 1, pp. 136.

Remarks

If you are interested in this method, please contact a SCIEX sales representative for further information. Method is shared after confidentiality agreement has been signed, and installation of the method will be coordinated by an application support specialist.

Table 2. List of components in the MRM library

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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