Extinction Coefficient of Protein

By UV-absorption (A280) and quantitative amino acid analysis

Order 12345

John Doe

ABCDE Pharma Inc.

Analysis start date: January 13, 2020

Analysis reporting date: January 27, 2020

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Samples received

The following sample was received at Alphalyse for protein analysis.

Sample 1

Objective

Determination of the protein molar extinction coefficient by absorbance measurement (A280) of a dilution series with known protein concentrations. The accurate protein concentration is measured by quantitative amino acid analysis.

Results Summary

AMINO ACID ANALYSIS

The concentration of the sample was determined by quantitative Amino Acid Analysis (AAA).

Sample	Analysis 1 [ug/uL]	Analysis 2 [ug/uL]	Analysis 3 [ug/uL]	Average [ug/uL]
Sample 1	2.30	2.32	2.30	**2.31**

MOLAR EXTINCTION COEFFICIENT and ABSORPTIVITY CONSTANT

Sample	Absorptivity Constant (L/g · cm)	Molar extinction coefficient (L/mol·cm)	A280 measurement range
Sample 1	1.7061	226768	0.2-1.6

When using the absorptivity constant for concentration measurements of samples, the following conditions should be applied:

-The buffer should be: ABCDE Pharma Inc. formulation buffer.
-The temperature should be 23°C (room temperature).
-The absorbance should be within the A280 measurement range.

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Analytical Procedure

Introduction

AMINO ACID ANALYSIS
Amino acid analysis is a method to determine the absolute amounts of individual amino acids in a sample. The amino acids are separated and quantified using ion exchange chromatography and post-column derivatization with ninhydrin. The method can be applied to free amino acids, as well as to peptides and proteins after hydrolysis into free amino acids. The total amount of proteins with unknown sequences is calculated as the sum of the amino acids. For a purified protein with known sequence, the specific protein amount is calculated using the most stabile amino acids and the known amino acid composition.

DETERMINATION OF PROTEIN EXTINCTION COEFFICIENT & ABSORPTIVITY CONSTANT
The molar extinction coefficient of a protein can be determined by A280 absorbance measurement if the protein concentration and molecular weight is known.
Beer-Lambert law states that the molar absorptivity is constant, and that the absorbance is proportional to the concentration of the substance in a solvent and measured at a given wavelength. The Molar Absorptivity is commonly called the Molar Extinction Coefficient.

$A = \varepsilon \cdot l \cdot c$ where A is the absorbance at a given wave length (λ)
A Absorbance
l Light path (cm)
c Concentration (g/L)
ε Molar extinction coefficient (L/mol·cm)
When l = 1cm, then $A = \varepsilon \cdot c$

The specific absorptivity constant is determined as the slope of the line when the absorbance is plotted against the concentration. It is converted to the molar extinction coefficient by multiplying with the molecular weight (MW) of the protein. The MW can be calculated if the amino acid sequence is known, or alternatively determined experimentally by mass spectrometry.

CALCULATION OF PROTEIN CONCENTRATION
When the absorptivity constant (L/g·cm) has been determined for a particular protein in a specified buffer and temperature, the concentration (g/L) of that protein can be determined from its measured absorbance at A280 using a derivative of the Beer-Lambert law:

Protein concentration = Absorbance of protein in solution (1-cm path length) / absorptivity constant.
Note that the measured absorbance can be significantly influenced by the buffer composition, temperature, and pH of the solvent.

Experimental

AMINO ACID ANALYSIS BY ION EXCHANGE CHROMATOGRAPHY
The acid hydrolysis is performed for 20 hours at 110°C, in 6 M hydrochloric acid, 0.1% phenol, 0.1% thioglycolic acid. The hydrolysis takes place under reduced pressure in an atmosphere of nitrogen. Identification and quantification of the amino acids is performed on a BioChrom 30 amino acid analyzer using ion exchange chromatography, post-column derivatization with ninhydrin and detection at two wavelengths, 570 nm and 440 nm. A known amount of the unusual amino acid Sarcosine (Sar) is added as an internal control standard. 18 of the common

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20 amino acids are determined, since Tryptophan is degraded during the hydrolysis, and the yield of Cysteine is so variable that it is not calculated. The Asparagine is determined with Aspartic acid (Asx) and Glutamine with glutamic acid (Glx). The total protein content is calculated as the sum of the individual 18 amino acids.

For quantification of a pure protein with known amino acid sequence, the specific protein content in picomoles is calculated using the most stabile amino acids and the expected picomoles calculated from the known protein amino acid composition.

AMINO ACID ANALYSIS DATA INTERPRETATION
In the amino acid analysis, it should be noted that:
1.Serine and threonine are degraded slightly during acid hydrolysis, and recoveries can be 10% lower than expected.
2.Methionine can be oxidized during hydrolysis, usually less than 10% is oxidized.
3.Valine and isoleucine bonds (Val-Val, Ile-Val, Val-Ile, Ile-Ile) are difficult to hydrolyse and recoveries can be 5-15% lower than expected.
4.Glycine content is often higher than expected because it is a frequent contaminant due to its use in many buffers.
5.The results table contains the columns Raw pmol and Corrected pmol. The Corrected pmol is based on analysis of known amounts of the NIST BSA standard that is used to determine a Compensation factor to correct for differences in ninhydrin reactivity and hydrolysis efficiency (Data from the standard can be provided on request).

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Extinction Coefficient of Protein

Order 12345

SEQUENCE DATA USED FOR CALCULATIONS

Residue	#
A	55
C	34
D	45
E	57
F	37
G	126
H	23
I	28
K	73
L	91
M	10
N	55
P	80
Q	61
R	34
S	153
T	93
V	99
W	26
Y	52

MW: 132916.1 Da

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Extinction Coefficient of Protein

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Result - Amino Acid Analysis

Analysis 1

Order #	12345
AAA order #	123
Signal strength	Good
Appendix:	20200116_1_Method Report.pdf

	SEQUENCE INFO		ANALYSIS DATA		CALCULATIONS			
Amino acids	**Number of residues**	**Number of residues ex. Cys, Trp**	**Raw pmole**	**Corrected pmole**	**Calc. % of AA**	**Calc. number of AA**	**Deviation from sequence**	**Protein amount (pmole) calc. from sequence**
Asp/Asn	100	100	3276	3646	10.5	105.5	5.5	36.46
Thr	93	93	2833	3153	9.1	91.2	-1.8	33.90
Ser	153	153	4649	5174	14.9	149.7	-3.3	33.82
Sar								
Glu/Gln	118							
Gly	126	126	4409	4907	14.2	142.0	16.0	
Ala	55	55	2226	2478	7.1	71.7	16.7	
Cys	34							
Val	99	100	3238	3604	10.4	104.3	4.3	36.04
Met	10	10	152	169	0.5	4.9	-5.1	
Ile	28	28	788	877	2.5	25.4	-2.6	31.30
Leu	91	91	2896	3224	9.3	93.3	2.3	35.42
Tyr	52							
Phe	37	37	1038	1155	3.3	33.4	-3.6	31.21
His	23	23	751	836	2.4	24.2	1.2	36.34
Lys	73	73	2460	2738	7.9	79.2	6.2	37.50
Arg	34	34	499	555	1.6	16.1	-17.9	
Pro	80	80	1932	2150	6.2	62.2	-17.8	
Trp	26							
Total	**1233**	**1003**	**31146**	**34664**	**100**	**1003**		
Average								**34.67**

Compensation factor	1.11
Loaded on instrument (µL)	2
MW (g/mol)	132916.1
Amount in sample (µg/µl)	**2.30**

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Extinction Coefficient of Protein

Order 12345

Analysis 2

Order #	12345
AAA order #	123
Signal strength	Good
Appendix:	20200116_3_Method Report.pdf

	SEQUENCE INFO		ANALYSIS DATA		CALCULATIONS			
Amino acids	**Number of residues**	**Number of residues ex. Cys, Trp**	**Raw pmole**	**Corrected pmole**	**Calc. % of AA**	**Calc. number of AA**	**Deviation from sequence**	**Protein amount (pmole) calc. from sequence**
Asp/Asn	100	100	3263	3631	10.4	104.5	4.5	36.31
Thr	93	93	2834	3154	9.1	90.8	-2.2	33.92
Ser	153	153	4617	5139	14.7	147.9	-5.1	33.59
Sar								
Glu/Gln	118							
Gly	126	126	4384	4879	14.0	140.5	14.5	
Ala	55	55	2173	2418	6.9	69.6	14.6	
Cys	34							
Val	99	100	3246	3612	10.4	104.0	4.0	36.12
Met	10	10	149	165	0.5	4.8	-5.2	
Ile	28	28	824	918	2.6	26.4	-1.6	32.77
Leu	91	91	2956	3290	9.4	94.7	3.7	36.16
Tyr	52							
Phe	37	37	1134	1262	3.6	36.3	-0.7	34.11
His	23	23	748	833	2.4	24.0	1.0	36.21
Lys	73	73	2456	2734	7.8	78.7	5.7	37.45
Arg	34	34	494	550	1.6	15.8	-18.2	
Pro	80	80	2026	2254	6.5	64.9	-15.1	
Trp	26							
Total	**1233**	**1003**	**31305**	**34841**	**100**	**1003**		
Average								**35.18**

Compensation factor	1.11
Loaded on instrument (µL)	2
MW (g/mol)	132916.1

Amount in sample (µg/µl)	**2.32**

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Extinction Coefficient of Protein

Order 12345

Analysis 3

Order #	12345
AAA order #	123
Signal strength	Good
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	SEQUENCE INFO		ANALYSIS DATA		CALCULATIONS			
Amino acids	**Number of residues**	**Number of residues ex. Cys, Trp**	**Raw pmole**	**Corrected pmole**	**Calc. % of AA**	**Calc. number of AA**	**Deviation from sequence**	**Protein amount (pmole) calc. from sequence**
Asp/Asn	100	100	3265	3633	10.5	105.4	5.4	36.33
Thr	93	93	2801	3118	9.0	90.4	-2.6	33.52
Ser	153	153	4566	5082	14.7	147.4	-5.6	33.22
Sar								
Glu/Gln	118							
Gly	126	126	4376	4870	14.1	141.2	15.2	
Ala	55	55	2154	2397	6.9	69.5	14.5	
Cys	34							
Val	99	100	3228	3593	10.4	104.2	4.2	35.93
Met	10	10	158	176	0.5	5.1	-4.9	
Ile	28	28	795	885	2.6	25.7	-2.3	31.61
Leu	91	91	2946	3278	9.5	95.1	4.1	36.03
Tyr	52							
Phe	37	37	1091	1215	3.5	35.2	-1.8	32.83
His	23	23	740	824	2.4	23.9	0.9	35.82
Lys	73	73	2458	2735	7.9	79.3	6.3	37.47
Arg	34	34	469	522	1.5	15.1	-18.9	
Pro	80	80	2027	2256	6.5	65.4	-14.6	
Trp	26							
Total	**1233**	**1003**	**31075**	**34585**	**100**	**1003**		
Average								**34.75**

Compensation factor	1.11
Loaded on instrument (µL)	2
MW (g/mol)	132916.1
Amount in sample (µg/µl)	**2.30**

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Extinction Coefficient

A280 MEASUREMENTS

Conc [mg/ml]	Abs
0.9000	1.638
0.9000	1.628
0.9000	1.640
0.7500	1.364
0.7500	1.363
0.7500	1.362
0.5000	0.935
0.5000	0.933
0.5000	0.938
0.2500	0.456
0.2500	0.463
0.2500	0.465
0.1250	0.235
0.1250	0.236
0.1250	0.233

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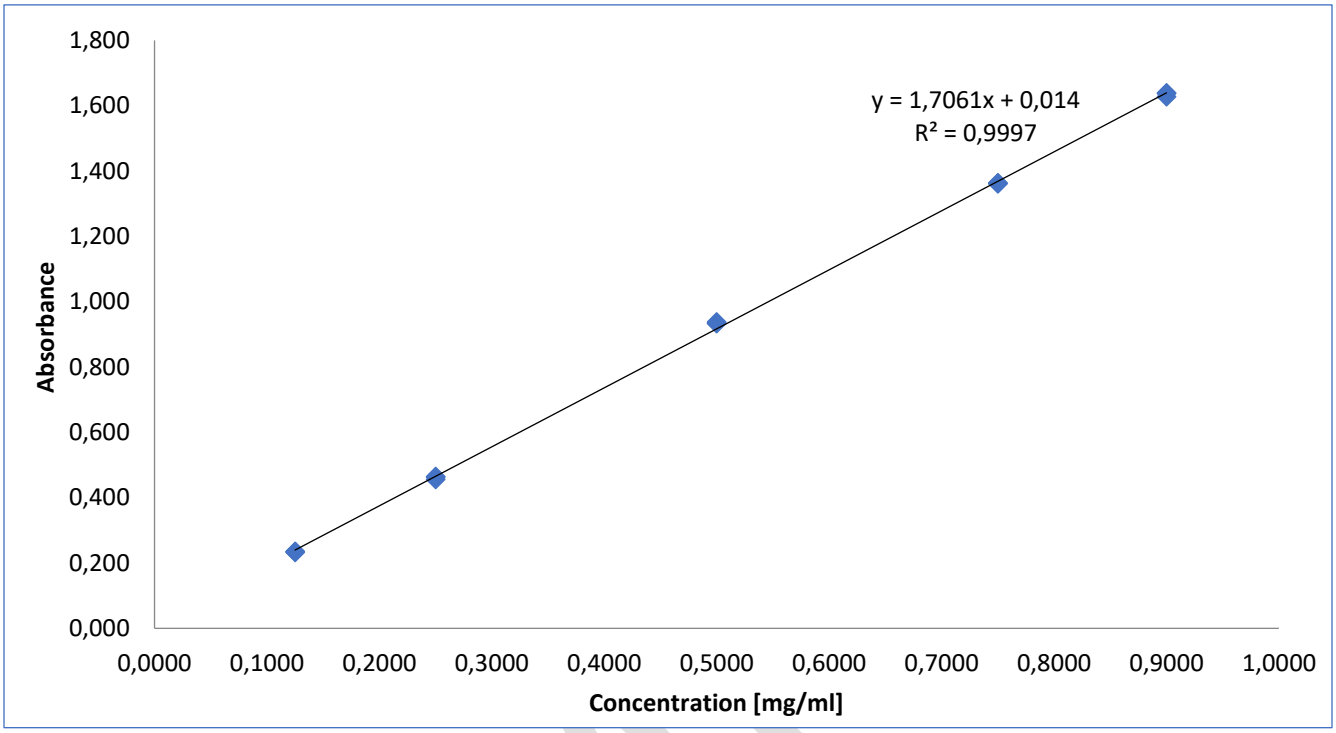
Order 12345

CALIBRATION CURVE AND CALCULATIONS



Conc [mg/ml]	Abs	Conc. calculated	Accuracy (%)	RSD%
0.9000	1.638	0.899	-0.1	
0.9000	1.628	0.894	-0.7	
0.9000	1.640	0.900	0.0	0.38
0.7500	1.364	0.748	-0.3	
0.7500	1.363	0.747	-0.4	
0.7500	1.362	0.746	-0.5	0.08
0.5000	0.935	0.510	2.0	
0.5000	0.933	0.509	1.8	
0.5000	0.938	0.512	2.3	0.26
0.2500	0.456	0.245	-2.1	
0.2500	0.463	0.249	-0.5	
0.2500	0.465	0.250	-0.1	1.03
0.1250	0.235	0.122	-2.1	
0.1250	0.236	0.123	-1.7	
0.1250	0.233	0.121	-3.0	0.66

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The calibration curve was linear in the complete measurement range (R2 > 0.99). The accuracy of all the calibration points were below 15% ±. The relative standard deviation percentage (RSD%) of the triplicate measurements were all below 15%.

The calibration curve was accepted and the slope could be used for determination of the molar extinction coefficient.

DETERMINATION OF ABSORPTIVITY CONSTANT AND MOLAR EXTINCTION COEFFICIENT

Absorptivity constant as = slope of calibration curve = 1.7061 L/g · cm
Molar extinction coefficient ε = as · MW = 1.7061 L/g · cm · 132916.1 g/mol = 226768 L/mol · cm

The samples to be measured must be diluted to an absorbance between 0.2-1.6 at 280nm, since this measurement range was proven to be linear.

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