Application Note

Bovine serum albumin (BSA) and thyroglobulin physical reference standards for molecular weight and polydispersity determinations using SEC-MALS

usp Biologics™

Introduction

Development and manufacturing of conjugated vaccines requires characterization of their molecular weight, which is a critical quality attribute (CQA) of the immunogen, and is correlated with the level of immune response. For biotherapeutics, the presence of high molecular weight (HMW) aggregates must also be determined, as these are impurities and can affect efficacy leading to an increased risk of immunogenicity.

Example: Characterization of monovalent conjugates using SEC-MALS

Pneumococcal capsular polysaccharides are made up of hundreds of highly charged repeating units, each with a unique chemical structure that contributes to its immunogenic and physiochemical properties. Conjugate vaccines are thus likely be heterogeneous with polydisperse forms in various association states and molecular weights ranging from 1 – 10 MDa. The example conjugate vaccine consists of polysaccharides and a carrier protein such as Cross-Reacting Material 197 (CRM197). The combination of these factors complicates their characterization and absolute molecular weight determination. **Figure 1** provides an example of molecular weight distribution of a conjugate vaccine as measured by size-exclusion chromatography coupled with multi-angle light scattering (SEC-MALS).[1]

SEC-MALS is considered the gold standard method for molecular weight determination and characterization of conjugate vaccines. SEC-MALS provides the sensitivity needed to detect changes in molecular association/entanglement states of a polymeric material such as a conjugate vaccine. SEC-MALS is also used for biochemical and structural analyses of other large, complex proteins including tumor suppressor proteins, antibodies, and virus-like particles (VLPs).

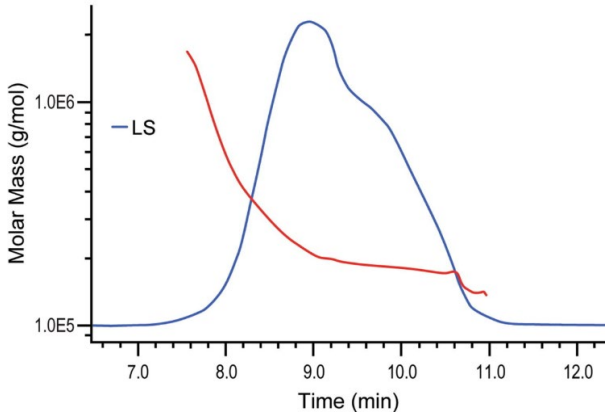


Figure 1. Molecular weight distribution of a conjugate vaccine as measured by SEC. Blue line: light scattering; red line: molar mass

The need for reference standards

It is essential to use a calibrant for SEC-MALS to align and normalize light scattering versus molar mass determination. Proteins such as bovine serum albumin (BSA) or human serum albumin (HSA) are commonly used for this purpose. Additionally, system suitability is required for Good Manufacturing Practice (GMP); BSA, HSA and thyroglobulin can be used to bracket the article of interest. BSA and HSA have a molecular weight of ~66 kDa, while thyroglobulin has a molecular weight of ~660 kDa.

Given the size distribution of conjugate vaccines and other biologics, it is critical to incorporate standards that bracket the target test article molecular weight into the SEC-MALS analysis. Well-characterized BSA and thyroglobulin have precise molecular weights and can be used with confidence as reference standards to support the determination of molecular weight and polydispersity of conjugate vaccines and complex proteins by SEC-MALS. In addition, the

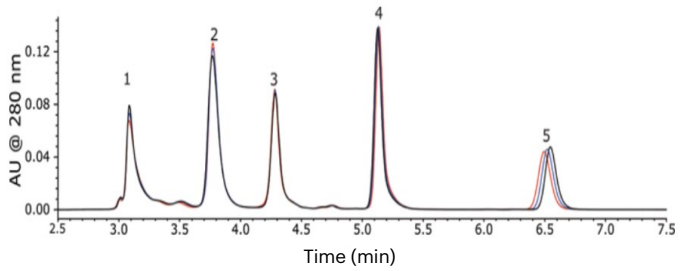


Figure 2. SEC separation of thyroglobulin (1), IgG (2), BSA (3), myoglobin (4), and uracil (5).

solubility and stability of BSA and thyroglobulin enhance reproducible and consistent outcomes, improving the accuracy of size calculations. **Figure 2** shows an overlay chromatogram of the separation by SEC of thyroglobulin, IgG, BSA, myoglobulin, and uracil.[2]

These standards are essential when using SEC-MALS to analyze the CQAs of conjugate vaccines, including the purity of protein carriers prior to conjugation, proof of conjugation, and the stability of the conjugated vaccine.[3]

USP Reference Standards for BSA and thyroglobulin

To support the analysis and characterization of monovalent conjugates (MVCs) using SEC-MALS, USP offers the reference standards shown in **Table 1**. Amino acid sequences for both reference standards are provided in the respective reference standard certificates.

Size variant assessment by SE-HPLC

The USP Molar Mass Determination Reference Standards (BSA or Thyroglobulin) were evaluated using the SE-HPLC method outlined in **Table 2** to determine the monomer peak, in addition to any high and low molecular weight species (BSA: **Figure 3**, Thyroglobulin: **Figure 4**). To determine the molecular weight accurately with good separation, one guard column and three SEC columns with different pore sizes are utilized to optimize the test method.

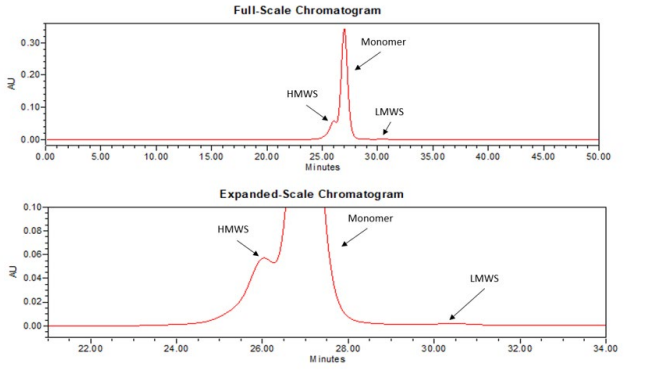
Molecular weight and polydispersity determination by SEC-MALS

The USP Molar Mass Determination Reference Standards (BSA or thyroglobulin) were also evaluated using the SEC-MALS method outlined in **Table 3** to determine molecular weight and polydispersity (BSA: **Figure 5**, Thyroglobulin: **Figure 6**).

It is important to note that if the same columns are used as specified in the USP Reference Standard certificates, the corresponding SE-HPLC and SEC-MALS chromatograms can serve as examples of what should be expected. If different columns are used for other applications such as the study

Table 2. SE-HPLC method parameters

Samples:	5 mg/mL USP BSA for Molar Mass Determination Reference Standard, OR 5 mg/mL USP Thyroglobulin for Molar Mass Determination Reference Standard
Columns:	Guard column: TSK gel GS2500WxlGuard 6 .0 mm x 4 cm, 12 µm Analytical columns: TSK G6000OWXL 7.8 mm x 300 mm, 13 µm, L39 TSK G5000PWXL 7.8 mm x 300 mm, 10 µm, L39 TSK G4000PWXL 7.8 mm x 300 mm, 10 µm, L39
Detector:	UV 280 nm
Mobile Phase:	10 mM Sodium Chloride, 200 µg/mL Sodium Azide
Elution:	Isocratic
Method:	Injection volume: 100 µL Flow rate: 1.0 mL/min Column temperature: 35 °C Sampler temperature: 5 °C

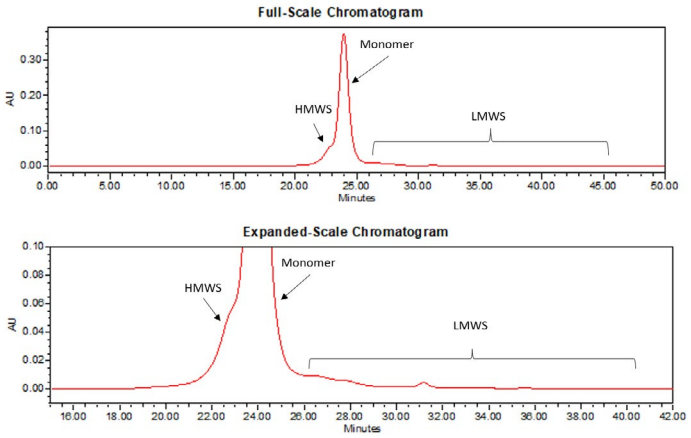


High Molecular Weight (HMWS)	Monomer	Low Molecular Weight (LMWS)
14.2 %	85.5 %	0.3 %

Figure 3. Separation of USP BSA for Molar Mass Determination Reference Standard monomer and high molecular weight species (HMWS) and low molecular weight species (LMWS) by SEC.

Table 1. USP BSA and thyroglobulin reference standards for Molar Mass Determination

USP Reference Standard	Catalog #	CAS #	Mass	Extinction Coefficient
BSA for Molar Mass Determination[4]	1076103	9048-46-8	~66 kDa	43,824 mL mg^{-1} cm^{-1}
Thyroglobulin for Molar Mass Determination[5]	1666032	9010-34-8	~660 kDa	654,560 mL mg^{-1} cm^{-1}



High Molecular Weight (HMWS)	Monomer	Low Molecular Weight (LMWS)
10.5%	83.1%	6.5%

Note: The sum of HMWS, monomer and LMWS is 100.1 due to rounding.

Figure 4. Separation of USP Thyroglobulin for Molar Mass Reference Standard monomer and high molecular weight species (HMWS) and low molecular weight species (LMWS) by SEC.

Table 3. SEC-MALS method parameters.

Sample:	5 mg/mL USP BSA for Molar Mass Determination Reference Standard, OR 5 mg/mL USP Thyroglobulin for Molar Mass Determination Reference Standard
Columns:	Guard column: TSK gel GS2500WxlGuard 6 .0 mm x 4 cm, 12 µm Analytical columns: TSK G6000OWXL 7.8 mm x 300 mm, 13 µm, L39 TSK G5000PWXL 7.8 mm x 300 mm, 10 µm, L39 TSK G4000PWXL 7.8 mm x 300 mm, 10 µm, L39
Detectors:	SEC: UV 280 nm MALS: Laser
Mobile Phase:	10 mM Sodium Chloride, 200 µg/mL Sodium Azide
Elution:	Isocratic
Method:	Injection volume: 100 µL Flow rate: 1.0 mL/min MALS temperature: room temperature RI temperature: 35 °C dn/dc: 0.185 mL/g

of large proteins, anti-drug antibodies, or VLPs, the eluted retention time may not match that of the USP Reference Standard. These USP Reference Standards can still serve as examples of the distribution of sizes, molecular weight, and polydispersity by SEC-MALS, regardless of the retention time.

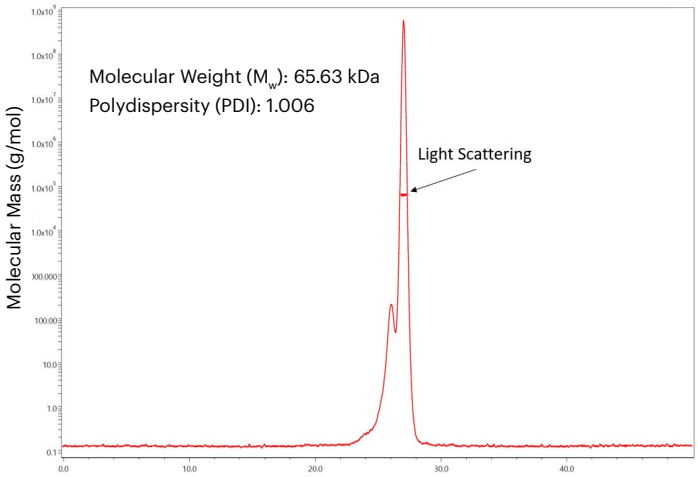


Figure 5. Molecular weight and polydispersity determination of USP BSA for Molar Mass Reference Standard by SEC-MALS.

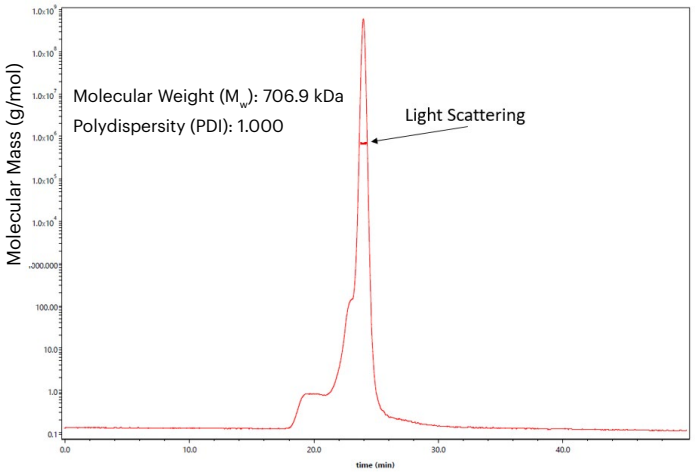


Figure 6. Molecular weight and polydispersity determination of USP Thyroglobulin for Molar Mass Reference Standard by SEC-MALS.

Identity determination using peptide mapping by mass spectrometry (MS/MS)

The identities of the USP Molar Mass Determination Reference Standards (BSA or thyroglobulin) were confirmed using peptide mapping with sequence coverage. The tests were performed using trypsin enzyme digestion. A representative peptide sequence coverage is shown for BSA in **Figure 7** and thyroglobulin in **Figure 8**; the average sequence coverage of two sample injections for BSA was 100% and for thyroglobulin was 92%.

BSA Coverage: 100%

1: 1 to 80	DTHKSEIAHR	FKDLGEEHFK	GLVLIAFSQY	LQQCPFDEHV	KLVNELTEFA	KTCVADESHA	GCEKSLHTLF	GDELCKVASL	
1: 81 to 160	RETYGDMADC	CEKQEPERNE	CFLSHKDDSP	DLPKLKPDPN	TLCDEFKADE	KKFWGKYLYE	IARRHPYFYA	PELLYYANKY	
1: 161 to 240	NGVFQECCQA	EDKGACLLPK	IETMREKVLT	SSARQRLRCA	SIQKFGERAL	KAWSVARLSQ	KFPKAEFVEV	TKLVTDLTKV	
1: 241 to 320	HKECCHGDLL	ECADDRADLA	KYICDNQDTI	SSKLKECCDK	PLLEKSHCIA	EVEKDAIPEN	LPPLTADFAE	DKDVCKNYQE	
1: 321 to 400	AKDAFLGSFL	YEYSRRHPEY	AVSVLLRLAK	EYEATLEECC	AKDDPHACYS	TVFDKLKHLV	DEPQNLIKQN	CDQFEKLGEY	
1: 401 to 480	GFQNALIVRY	TRKVPQVSTP	TLVEVSRSLG	KVGTRCCTKP	ESERMPCTED	YLSLILNRLC	VLHEKTPVSE	KVTKCCTESL	
1: 481 to 560	VNRRPCFSAL	TPDETYVPKA	FDEKLFTFHA	DICTLPDTEK	QIKKQTALVE	LLKHKPKATE	EQLKTVMENF	VAFVDKCCAA	
1: 561 to 583	DDKEACFAVE	GPKLVVSTQT	ALA						

Figure 7. Representative peptide sequence coverage of USP BSA for Molar Mass Reference Standard.

Thyro Coverage: 93%

1: 1 to 80	MALALWVFGL	LDLICLASAN	IFEYQVDAQP	LRPCELQRER	AFLKREDYVP	QCAEDGSFQT	VQCGKDGASC	WCVDADGREV	
1: 81 to 160	PGSRQPGRPA	ACLSFCQLQK	QQILLSSYIN	STATSYLPQC	QDSGDYSPVQ	CDLRRRQCWC	VDAEGMEVYG	TRQQGRPARC	
1: 161 to 240	PRSCEIRNRR	LLHGVGDRSP	PQCSPDGAFR	PVQCKLVNTT	DMMIFDLVHS	YSRFPDAFVT	FSSFRSRFPE	VSGYCYCADS	
1: 241 to 320	QGRELAETGL	ELLLDEIYDT	IFAGLDLAST	FAETTLYRIL	QRRFLAVQLV	ISGRFRCPTK	CEVERFAATS	FRHPYVPSCH	
1: 321 to 400	PDGEYQAAQC	QQGGPCWCVD	SRGQEIPGTR	QRGEPPSCAE	DQSCPSERRR	AFSRLRFGPS	GYFSRRSLLL	APEEGPVSQR	
1: 401 to 480	FARFTASCPP	SIKELFLDSG	IFQPMLQGRD	TRFVAPESLK	EAIRGLFPSR	ELARLALQFT	TNAKRLQQNL	FGGRFLVKVG	
1: 481 to 560	QFNLSGALGT	RGTFNFSHFF	QQLGLPGFQD	GRALADLAKP	LSVGLNSNPA	SEAPKASKID	VALRKPVVGS	FGFEVNLQEN	
1: 561 to 640	QNALQFLSSF	LELPEFLLFL	QHAISVPEDI	ARDLGDVMEM	VFSSQGCGQA	PGSLFVPACT	AEGSYEEVQC	FAGDCWCVDA	
1: 641 to 720	QGRELAGSRV	RGGRPRCPTE	CEKQRARMQS	LLGSQPAGSS	LFVPACTSKG	NFLPVQCFNS	ECYCVDTEGQ	PIPGTRSALG	
1: 721 to 800	EPKKCPSPCQ	LQAERAFLGT	VRTLVSNPST	LPALSSIYIP	QCSASGQWSP	VQCDGPPEQA	FEWYERWEAQ	NSAGQALTPA	
1: 801 to 880	ELLMKIMSYR	EAASRNFRLF	IQNLYEAGQQ	GIFPGLARYS	SFQDVPVSVL	EGNQTQPGGN	VFLEPYLFWQ	ILNGQLDRYP	
1: 881 to 960	GPYSDFSAPL	AHFDLRSCWC	VDEAGQKLEG	TRNEPNKVPA	CPGSCEEVKL	RVLQFIREAE	EIVTYSNSSR	FPLGESFLAA	
1: 961 to 1040	KGIRLTDEEL	AFPPLSPSRE	TFLEKFLSGS	DYAIRLAAQS	TFDFYQRRLV	TLAESPRAPS	PVWSSAYLPQ	CDAFGGWEPV	
1: 1041 to 1120	QCHAATGHCW	CVDGKGEYVP	TSLTARSRQI	PQCPTSCERL	RASGLLSSWK	QAGVQAEPSP	KDLFIPTCLE	TGEFARLQAS	
1: 1121 to 1200	EAGTWCVDPA	SGEGVPPGTN	SSAQCPSLCE	VLQSGVPSRR	TSPGYSPACR	AEDGGFSPVQ	CDPAQGSCWC	VLGSGEEVPG	
1: 1201 to 1280	TRVAGSQPAC	ESPQCPLPFS	VADVAGGAIL	CERASGLGAA	AGQRCQLRCS	QGYRSAFPPE	PLLCSVQRRR	WESRPPQPRA	
1: 1281 to 1360	CQRPQFWQTL	QTQAQFQLLL	PLGKVCSADY	SGLLLAFQVF	LLDELTARGF	CQIQVKTAGT	PVSIPVCDDS	SVKVECLSRE	
1: 1361 to 1440	RLGVNITWKL	QLVDAPPASL	PDLQDVEEAL	AGKYLAGRFA	DLIQSGTFQL	HLDSKTFSAD	TSIRFLQGDR	FGTSPRTQFG	
1: 1441 to 1520	CLEGFGRVVA	ASDASQDALG	CVKCPEGSYF	QDEQCIPCPA	GFYQEQAGSL	ACVPCPEGRT	TVYAGAFSQT	HCVTDCQKNE	
1: 1521 to 1600	VGLQCDQDSQ	YRASQRDRTS	GKAFCVDGEG	RRLPWTEAEA	PLVDAQCLVM	RKFEKLPESK	VIFSADVAVM	VRSEVPGSES	
1: 1601 to 1680	SLMQCLADCA	LDEACGFLTV	STAGSEVSCD	FYAWASDSIA	CTTSGRSEDA	LGTSQATSFG	SLQCQVKVRS	REGDPLAVYL	
1: 1681 to 1760	KKGQEFTITG	QKRFEQTGFQ	SALSGMYSPV	TFSASGASLA	EVHLFCLLAC	DHDSCCDGFI	LVQVQGGPLL	CGLLSSPDVL	
1: 1761 to 1840	LCHVRDWRDP	AEAQANASCP	GVTYDQDSRQ	VTLRLGGQEI	RGLTPLEGTQ	DTLTSFQQVY	LWKDSDMGSR	SESMGCRRDT	
1: 1841 to 1920	EPRPASPSET	DLTTGLFSPV	DLIQVIVDGN	VSLPSQQHWL	FKHLFSLQQA	NLWCLSRCAG	EPSFCQLAEV	TDSEPLYFTC	
1: 1921 to 2000	TLYPEAQVCD	DILESSPKGC	RLILPRRPSA	LYRKKVVLQD	RVKNFYNRLP	FQKLTGISIR	NKVPMSDKSI	SSGFFECERL	
1: 2001 to 2080	CDMDPCCTGF	GFLNVSQLKG	GEVTCLTLNS	LGLQTCSEEY	GGVWRILDCG	SPDTEVRTYP	FGWYQKPVSP	SDAPSFCPSV	
1: 2081 to 2160	ALPALTENVA	LDSWQSLALS	SVIVDPSIRN	FDVAHISTAA	VGNFSAARDR	CLWECSRHQD	CLVTTLQTQP	GAVRCMFYAD	
1: 2161 to 2240	TQSCTHSLQA	QNCRLLLHEE	ATYIYRKPNI	PLPGFGTSSP	SVPIATHGQL	LGRSQAIQVG	TSWKPVDQFL	GVPYAAPPLG	
1: 2241 to 2320	EKRFRAPEHL	NWTGSWEATK	PRARCWQPGI	RTPTPPGVSE	DCLYLNVFVP	QNMAPNASVL	VFFHNAAEGK	GSGDRPAVDG	
1: 2321 to 2400	SFLAAVGNLI	VVTASYRTGI	FGFLSSGSSE	LSGNWGLLDQ	VVALTWVQTH	IQAFGGDPRR	VTLAADRGGA	DIASIHLVTT	
1: 2401 to 2480	RAANSRLFRR	AVLMGGSALS	PAAVIRPERA	RQQAAALAKE	VGCPSSSVQE	MVSCLRQEPA	RILNDAQTKL	LAVSGPFHYW	
1: 2481 to 2560	GPVVDGQYLR	ETPARVLQRA	PRVKVDLLIG	SSQDDGLINR	AKAVKQFEES	QGRTSSKTAF	YQALQNSLGG	EAADAGVQAA	
1: 2561 to 2640	ATWYYSLEHD	SDDYASFSRA	LEQATRDYFI	ICPVIDMASH	WARTVRGNVF	MYHAPESYSH	SSLELLTDVL	YAFGLPFYPA	
1: 2641 to 2720	YEGQFTLEEK	SLSLKIMQYF	SNFIRSGNPN	YPHEFSRRAP	EFAAPWPDFV	PRDGAESYKE	LSVLLPNRQG	LKKADCSFWS	
1: 2721 to 2769	KYIQSLKASA	DETKDGPSAD	SEEEDQPAGS	GLTEDLLGLP	ELASKTYSK				

Figure 8. Representative peptide sequence coverage of USP Thyroglobulin for Molar Mass Reference Standard

Conclusion

Some bacterial vaccines contain conjugate molecules that can have a wide range of molecular weights and exist in various association states, leading to difficulty with their characterization and reporting of this CQA. SEC-MALS is an analytical technique that overcomes this challenge and ensures that the vaccine conjugate is in the appropriate size range correlating to clinical efficacy.

The USP BSA for Molar Mass Determination Reference Standard and the USP Thyroglobulin for Molar Mass Determination Reference Standard support molecular weight and polydispersity determinations by SEC-MALS as system suitability standards for not only sizing of vaccine conjugates but also for other higher molecular weight, complex materials. Use of these established standards will help increase the confidence in analytical results and will help facilitate regulatory compliance.

References

1. Deng, J.Z.; Lin, J.; Chen, M.; Lancaster, C.; Zhuang, P. Characterization of High Molecular Weight Pneumococcal Conjugate by SEC-MALS and AF4-MALS. Polymers 2022, 14, 3769. doi.org/10.3390/polym14183769

2. Hong P, Koza S, Bouvier ESP. A review of size exclusion chromatography for the analysis of protein biotherapeutics and their aggregates. Journal of Liquid Chromatography & Related Technologies. 2012. 35:20, 2923-2950. doi: 10.1080/10826076.2012.743724

3. Micoli F, Adamo R, Costantino P. Protein carriers for glycoconjugate vaccines: History, selection criteria, characterization, and new trends. Molecules. 2018. 23:1451. doi: 10.3390/molecules23061451

4. USP Reference Standard Certificate for Bovine Serum Albumin for Molar Mass Determination.

5. USP Reference Standard Certificate for Thyroglobulin for Molar Mass Determination.

Available and upcoming resources on the utilization of polysaccharides by SEC-MALS

A general chapter describing the use of SEC-MALS to determine the molecular weight of polysaccharides in vaccines is currently in development. Entitled "<316> *Molar Mass Determination of Bacterial Polysaccharides Used in Human Vaccines by HPSEC-MALS*", the chapter describes a method using the same columns and parameters described above. Pullulans, BSA, and rAlbumin human reference standards applications, will be recognized in the USP General Chapter. The molecular weights of reference standards are determined by the official tests, procedures, and acceptance criteria incorporated in the general chapters.

Related USP Reference Standards

1. Bovine Serum Albumin (BSA) for Molar Mass Determination, Item number 1076103
2. Cross-Reacting Material 197 (CRM197), Item number 1150800
3. Recombinant Human Albumin (rAlbumin Human), Item number 1012595
4. Thyroglobulin for Molar Mass Determination, Item number 1666032

Pullulan Reference Standards (Coming Soon)

1. Pullulan for Molar Mass Determination (100kDa), Item number 1581781
2. Pullulan for Molar Mass Determination (400kDa), Item number 1581782
3. Pullulan for Molar Mass Determination (800kDa), Item number 1581780

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