

Accelerated Predictive Stability of Oligonucleotides using ASAP^{prime}®: A Case Study Using Waters' Lipid-Conjugated Antisense Oligonucleotide Reference Standard

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ABSTRACT

A case study was carried out that confirms the applicability of ASAP^{prime}® for accurate determination of shelf life of lyophilized oligonucleotides using a lipid-conjugated antisense oligonucleotide reference standard from Waters Corporation. This oligonucleotide was exposed to a range of statistically designed conditions, with the resulting purity data found to be well fit using the ASAP^{prime}® process of combining isoconversion times (times to hit the specification limit of 10% loss of purity) with a moisture-modified Arrhenius equation which accounts for both temperature and relative humidity impact.

INTRODUCTION

Oligonucleotides are a growing class of drug molecules that are based on nucleic acids¹. As with most biological molecules, their stability is sensitive to environmental factors such as temperature, humidity, and oxygen. Lyophilization (freeze drying) is a widely used technique to improve the stability and shelf life of drug products by removing the majority of the water, thereby reducing molecular mobility and corresponding reactivity.

The Accelerated Stability Assessment Program (ASAP) has been successfully applied to numerous small molecule, peptide, and biological drug products²⁻⁶. In the ASAP process, samples are stressed at a range of temperatures and relative humidities outside of packaging for up to one month, and the isoconversion time (i.e., time to reach the failure point) is determined at each condition. The isoconversion times are used to calculate the temperature and

moisture sensitivities of degradation using a moisture-modified Arrhenius equation (Eq. 1).

$$\ln k = \ln A - \frac{E_a}{RT} + B(RH) \quad (1)$$

Here, k is the isoconversion rate constant (i.e., the inverse of the time to fail), A is the collision frequency, E_a is the activation energy or temperature sensitivity, R is the gas constant, T is the absolute temperature, B is the moisture sensitivity, and RH is the equilibrium relative humidity to which the sample is exposed. Based on these parameters, the ASAP model is used to predict shelf life at the intended storage conditions and in different packaging configurations.

To verify that ASAP can be used to model the long-term stability behavior of oligonucleotides, FreeThink conducted an ASAP case study using Waters' antisense oligonucleotide (ASO) lipid-conjugated LC-MS reference standard. Purity loss was monitored using a hydrophilic interaction liquid chromatography (HILIC) UPLC method.

METHODS

Materials and Accelerated Stressing

A lipid conjugated ASO LC-MS reference standard (Part Number 186010747, **Table 1**) was provided by Waters Corporation as 1 nmol per vial. For each ASAP condition, one vial was placed open into a canning jar with a second vial containing the appropriate saturated salt for humidity control (**Table 2**). The canning jars were sealed and placed into the appropriate ovens in a staggered fashion such that all samples were removed at the end of the study. Control samples

were stored refrigerated and unopened for the duration of the study before being prepared for UPLC analysis along with stressed samples. All samples were analyzed in a preestablished random sequence to minimize any impact of chromatographic drift (systemic bias) during the analysis. Sample purity was calculated as a percentage of the total peak area.

Table 1. Oligonucleotide used in the ASAP case study

Component	Elemental Composition	Average Mass (Da)
16mer	5' d 5-Pal- [*] -MOE-MeC- [*] -MOE-G- [*] -MOE-MeC- [*] C*G*A* [*] T*A*A*G*G*T* [*] A*-MOE-MeC- [*] -MOE-A- [*] -MOE-MeC 3'	6050

* denotes PS bonds, MOE denotes methoxy ethyl 2' modifications, and MeC denotes a 5-methyl cytidine residue

Table 2. Stress conditions used for generating an ASAP stability model for the lipid-conjugated ASO

Temp (°C)	Saturated Salt	% RH	Time (d)
Control	NA	NA	0 (3 repeats)
25	LiCl	11	12
30	CsF	3	13, 21
35	KF	25	6, 21
40	LiCl	11	4, 9, 21
45	KF	21	2, 5, 16
50	LiCl	11	2, 5, 15
50	NaI	29	1

Chromatography

Stressed and control samples were reconstituted by adding 50 µL of 50:50 acetone:acetonitrile (ACN) and gently vortexing to ensure complete dissolution. A 15 µL aliquot of the resulting solution was transferred to a vial containing 985 µL of 50:50 acetone:ACN and mixed thoroughly. All samples were placed in an autosampler for analysis using an ACQUITY UPLC H-Class System with a Sample Manager with Flow Through Needle (SM-FTN) and a

GTxResolve Premier BEH Amide, 300 Å, 1.7 µm, 2.1 X 100 mm column under HILIC conditions⁷. The sample temperature was 5°C, the flow rate was 0.3 mL/min, and the needle wash was 50:50 ACN:H₂O. The detection wavelength was 260 nm. All data were acquired using Empower™ 3 software. The column temperature was set at 75°C and the injection volume was 10 µL. The Mobile Phases A and B were 90:10 and 10:90 ACN:100 mM ammonium acetate (v:v), respectively. The gradient was 0, 90:10; 0.67, 90:10; 4.67, 22:78; 5, 22:78; 5.33, 90:10; and 13.33 min, 90:10 A:B.

ASAPprime® Modeling

Chemical purity data for the oligonucleotide were modeled using ASAPprime® software (version 7.0). Calculations used 50,000 Monte Carlo simulations to determine error bars.

RESULTS AND DISCUSSION

Loss of purity in the lipid-conjugated ASO was modeled using a default fit (linear or bilinear if a single line has an R² < 0.90) with a standard deviation of all samples assumed to be 1% based on the reproducibility determined for the controls. Control samples had an average initial purity of 88%, so with an allowed decrease of 10% purity, a specification limit of 78% was used for the ASAP modeling. The fitting of these data at each condition is shown in **Figure 1**.

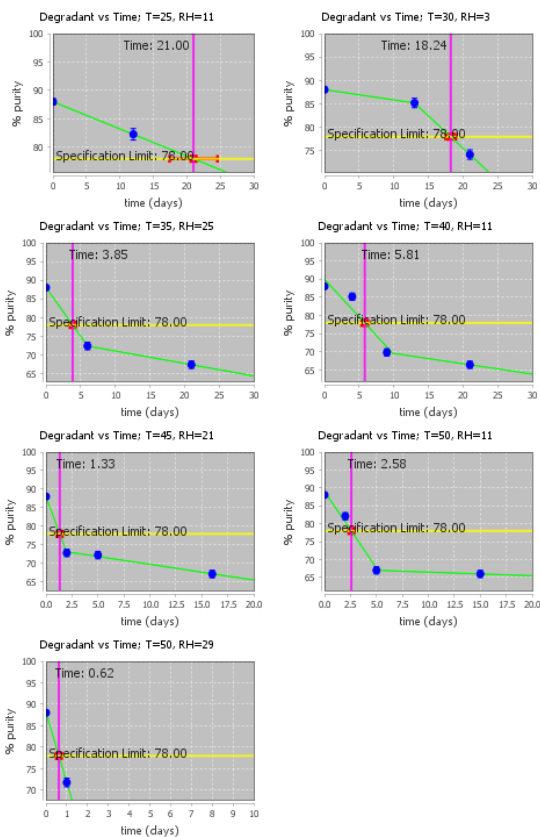


Figure 1. Plots for loss of purity in the lipid-conjugated ASO with a 78% specification limit (10% purity loss) indicated by the yellow line

The resulting fit parameters to the moisture-modified Arrhenius equation (Eq. 1) are given in **Table 3**. As can be seen, loss of purity is well fit by this equation, as evidenced by R^2 and Q^2 values of 0.982 and 0.945, respectively. Q^2 is the predicted residual error sum of squares (or PRESS R^2) and is a sensitive indicator of how well the fitted data predict points not used in the fitting⁸. The residuals plots of the model fit (**Figure 2**) indicate that the experimental isoconversion times (expressed as the logarithm of the inverse isoconversion times, $\ln k$) are close to those derived from the model.

Table 3. ASAPprime[®]-modeled Arrhenius parameters for loss of purity in the lipid-conjugated ASO to a specification limit of 78% (10% purity loss)

$\ln A$	E_a (kcal/mol)	B	R^2	Q^2
27.5	17.2 ± 2.2	0.066	0.982	0.945
± 3.7		± 0.011		

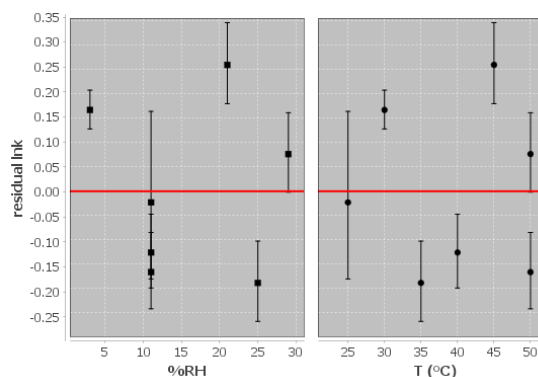


Figure 2. Residuals plots of fit to the moisture-modified Arrhenius model for loss of purity in the lipid-conjugated ASO to a specification limit of 78% (10% purity loss)

There is limited published information about the temperature and humidity dependence of oligonucleotide degradation. The International Conference on Harmonisation (ICH) uses an assumption that six months of storage at 25°C (accelerated conditions) is predictive of a two-year shelf life at 2–8°C. This equates to an E_a of 11.4 kcal/mol. Loss of purity in the lipid-conjugated ASO exhibits an E_a value of 17 kcal/mol. While this is lower than that of the average small molecule stability (27 kcal/mol), it is still higher than the ICH standard.

The high B term (0.07) versus the average 0.04 for small molecules indicates that degradation is significantly impacted by relative humidity. With lyophilized oligonucleotides often stored in impermeable (glass) packaging, the relative humidity is set by any residual moisture from the lyophilization process with no further moisture ingress. In this case, that residual moisture level

will have a large impact on the overall shelf life of the oligonucleotide.

Figure 3 shows the predicted long-term loss of purity for the lipid-conjugated ASO calculated at 5°C/1% RH and -20°C. It should be noted that for temperatures below water's freezing point (0C), relative humidity (i.e., the percent of saturated vapor pressure in the air) does not have a definition. As can be seen, the mean shelf life is six months at 5°C (refrigerated), while at -20°C (frozen), this oligonucleotide is predicted to be stable for more than three years.

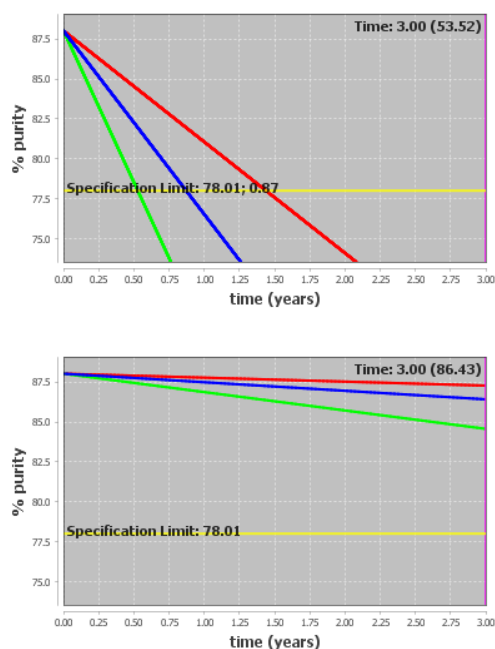


Figure 3. Predicted loss of purity of the lipid-conjugated ASO at constant 5°C/1% RH (top) and -20°C (bottom). The blue line represents the mean predicted behavior, with the green and red lines representing the 90% confidence interval (i.e., 95% probability that purity levels remain above the green line).

CONCLUSION

A three-week ASAP case study was conducted on an oligonucleotide lyophile to demonstrate that the chemical stability of RNA-based materials could be effectively modeled using the combination of isoconversion (time to hit the

specification limit) and a moisture-modified Arrhenius equation. The mathematical modeling shows with good statistics that loss of oligonucleotide purity is well-fit by a moisture-modified Arrhenius equation, supporting the use of ASAP for this class of molecules. Based on this case study, the lipid-conjugated ASO is predicted to exceed 10% purity loss after approximately six months at 5°C, even under low humidity conditions. This oligonucleotide therefore requires frozen (-20°C) storage to maintain acceptable purity. At that condition, the oligonucleotide is stable for more than three years.

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