REAL LIFE EVALUATION OF THE USE OF ACOUSTIC TUBES UNDER NORMAL OPERATIONS IN A COMPOUND MANAGEMENT DEPARTMENT

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Introduction

Evotec sample management team is responsible for the long-term storage and distribution of Evotec and partners compound collections. The collections are currently stored in micronics tubes and master plates at -20 °C and handled in a laboratory under humidity and temperature control. In 2019, Evotec decided to invest in acoustic tubes technology in order to support future customer need and plan for potential transfer of our libraries into acoustic tubes. In parallel to this decision, Evotec sample management decided to initiate an internal project to evaluate the impact of acoustic tubes usage on compound integrity. This poster highlights and describes the experiments performed, which substantiate and validate the use of acoustic tubes.

Materials & Methods

Samples / Compounds

Twenty molecules within five categories were carefully selected to represent compound behavior upon long term storage and use:

- Hardly soluble in DMSO
- Soluble in DMSO
- Photosensitive
- Oxidable
- Thermosensitive

All compounds used in this project were commercially available from Sigma-Aldrich and each category is made up of at least 3 molecules (see table 1). The tanimoto index was calculated (*Bajusz, D., Racz, A. & Héberger, K. Why is Tanimoto index an appropriate choice for fingerprint-based similarity calculations? J Cheminform 7, 20 (2015)*) (see table 2) in order to ensure that we have a high diversity at the molecules chosen per category.

Hardly soluble in DMSO	Soluble in DMSO	Photosensitive	Oxidable	Thermosensitive
Ciprofloxacin (85721-33-1)	Nifedipine (21829-25-4)	Nifedipine (21829-25-4)	2',7'-Dichlorofluorescein (76-54-0)	Resveratrol (501-36-0)
Colchicine (64-86-8)	5,5-Diphenylhydantoin (57-41-0)	(+)-Quinine (130-95-0)	Curcumin (458-37-7)	Lopinavir (192725-17-0)
Xanthozomicine (65277-42-1)	Indomethacin (53-86-1)	Ofloxacin (82419-36-1)	Dansylcadaverine (10121-91-2)	Fluvastine (867-26-3)
	Lidocaine (137-58-6)	Furosemide (54-31-9)		Quercetin 3-?-D-glucoside (482-35-9)
	Tetracaine (94-24-6)			Dexamethasone (50-02-2)

Category	Dissimilarity index mean
Soluble in DMSO	0.874
Hardly soluble in DMSO	0.869
Photosensitive	0.879
Thermosensitive	0.899
Oxidable	0.884
All categories	0.882

Table 1: Molecules used for the experiments grouped by categories

Table 2: Average dissimilarity index per compound category

Compounds preparation and labware

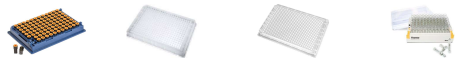
All compounds were dissolved using dimethyl sulfoxide (DMSO) (Merck, cat. #102931500).

Experiments 1 & 2
50 x 96 Tube racks were prepared, of each compound in triplicate, with a final volume of 70 µL. An additional six matrix racks, were prepared with a final volume of 650 µL of each compound also in triplicate, as controls for condition 1. The final concentration for all compounds was 10 mM; except for the molecule Ciprofloxacin, which was prepared at 1 mM due to an unexpected colloid formation in the preparation.

Experiment 3
Two compounds (Indomethacin & 5,5-Diphenylhydantoin) were dissolved in DMSO 100 % and DMSO 70 % to a final concentration of 40 mM in order to achieve 0.5 mM final necessary for the QC analysis.

Experiment 4
Eight compounds from three different categories were dissolved in DMSO 100% to a final concentration of 0.5 mM: Rifampicin, 5,5-Diphenylhydantoin, Nifedipine, Ofloxacin, Furosemide, 2',7'-Dichlorofluorescein, Curcumin and Dansylcadaverine.

Labware used



FluidX AcoustiX Sample (96TR) | Echo Qualified 384-Well Polypropylene (384PP) | Echo Qualified 384-Well Low Dead Volume (384LDV) | Thermo Scientific Matrix tubes (Matrix tubes)

Parameters calculation

The **total volume** and **DMSO percentage** of each sample were obtained using the Echo "survey". The **volume of water** and the **volume of DMSO** were calculated from the total volume and percentage of DMSO.
All the values obtained in the survey were normalized to initial values of total volume and DMSO percentage. To do so, an initial survey (in triplicate) of all the plates in the experiment was performed. Each time that a 96 tube rack is surveyed (in triplicate) during the experiments, for the values of each well or tube, the initial value was subtracted. The initial value for Experiment 1 correspond to t_0 and for Experiment 2 to the day of preparation (10-04-2020). In this way we are able to follow the changes of %DMSO and volume over time and under different conditions. To determine the **percentage of purity** (%Purity), for both 96 tube racks and matrix racks, 2 µL were transferred to a Greiner 784201 microplate and diluted to a final volume of 40 µL and final concentration of 0.5 mM. 1 µL of this was injected later into our UPLC device and the percentage purity was calculated proportionally to the set of peaks detected.

Instrumentation and chromatographic conditions

The survey to measure the total volume and DMSO percentage of each sample, was performed by using an Echo® 655T Liquid Handler by Beckman Coulter Life Sciences . An I-Class Acquity UPLC Waters / SQD 2 (Single Quadrupole mass Detector) Waters system equipped with an Acquity CSHC18 Column (2.1x50mm) 1.7 µm and Mass spectrometry ESI (Electrospray Ionization) was used. Wavelength was monitored at 210-400 nm and the injection volume was 1 µL. The sample was injected to a flow rate of 1 ml/min. The mobile phase was a mix of water, formic acid and acetonitrile (Phase A = H_2O + HCOOH 0.02 % pH 3, Phase B = CH_3CN + HCOOH 0.02%) with a gradient as in table 3.

Time (min)	A (%)	B (%)
0	95	5
2	0	100
2.5	0	100
2.6	95	5
3	95	5

Table 3: Mobile phase gradient

Data Analysis

The data was analyzed using R (https://www.R-project.org/) and R studio (Version 1.2.1335). The software used to run and analyze in the UPLC-MS data was MassLynx (Ver. 4.2) and Analytical Studio Professional (Ver 4.8) respectively. We have been supported by our Bio-statistical group.

Strategy plan summary

Experiment 1: Short-term evaluation (over 13Hrs) of DMSO hydration levels, total volume and volume of water and DMSO in microplates and acoustic tube racks, exposed under two different Evotec's environments: Condition 1: the usual condition in our laboratories (%Relative Humidity (RH) = 40-50; Temperature (ºC) = 22±3) and Condition 2: the environment present under our robot enclosures (%RH = 10-20; TºC = 22±3).
Experiment 2: Assessment of the effects of a short/long-term, -20 ºC / +21 ºC storage and freeze-thaw cycles on compound integrity and DMSO hydration levels.
Experiment 3: : Impact on sample transfer when a sample is hydrated. Compound concentration on hydrated solutions was compared with compound concentration on non hydrated solutions.
Experiment 4: Compound integrity assessment after repeated transfer with Echo.

Experiment 1 – DMSO hydration studies

Aim

The purpose of this experiment was to evaluate the DMSO hydration levels over a short period of time under normal operating conditions. Open laboratory conditions (condition 1) and Equipment-enclosure conditions (condition 2) were compared. In order to perform this experiment, repetitive surveys were performed to monitor 96TR, 384PP and 384LDV filled with 70 µL, 50 µL and 8 µL of compounds respectively. Microplates and tubes racks were exposed to both environments over a 13 hours period. Microplates and 96TR were then surveyed in two independent runs and each samples measured in triplicate.

Results

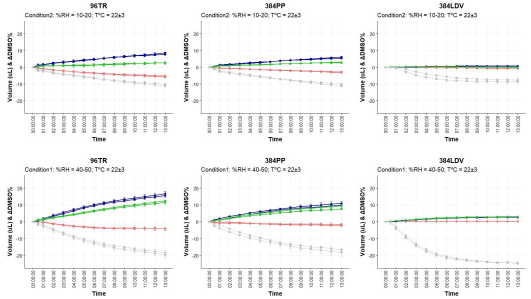


Figure 1: Average volume of water (in blue), the total volume (in green), volume of DMSO (in red) in µL and Δ DMSO% (DMSO% – DMSO% at t_0, in grey) in percentage over time is shown. 96TR, 384PP and 384LDV were initially filled with 70, 50 and 8 µL respectively and exposed to the environment for 13 Hrs. Error bars represent the standard deviation per experiment over the full microplate or tube rack.

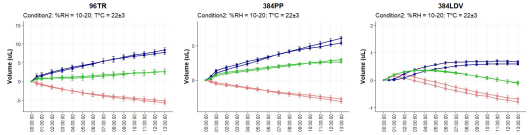


Figure 2: Same plot as figure 1, but the scale was changed, in order to visualize the evaporation (green points) occurring in 384LDV under enclosure conditions (condition 2)

Experiment 1 conclusions

- As could be expected, hydration levels of the compounds were higher for the open laboratory condition (condition 1) than for equipment enclosure conditions (condition 2). For the 96 tubes racks under laboratory conditions, the decrease of DMSO percentage within first two hours was about -4.7 % while for the equipment enclosure conditions, it was -3.2 %, approaching 15% (condition 1) and 8% (condition 2) after 13Hrs (see figure 1).
- We see little to no differences among chemical categories or among compounds with regards to the rate of water uptake; only Ofloxacin behaves differently from the rest of the compounds: It shows an increase in the rate of water uptake after 8-9 Hrs when exposed to the environment under laboratory conditions (not shown in the graphs) and is probably due to the hygroscopic nature of the molecule* (*ANSM)
- However we see differences in the hydration levels when comparing the different volumes: The lower the volume, the faster was the water uptake. Taking as reference, after 2 Hrs, the percentage of DMSO decrease for 8 µL was -9.1 % for laboratory conditions, while for 70 uL was only -4.7 % (see figure 1).
- No edge effects were observed in both conditions for microplates or rack tubes (data not shown)
- When focusing on analyzing evaporation rates, we measured very little evaporation for the total volume (70, 50 and 8 µL) during the 13 Hrs under laboratory conditions (condition 1), yet for the microplates 384LDV containing 8 µL, some evaporation is clearly visible after 3 Hrs under enclosure conditions (condition 2) (see figure 2, green points)

Experiment 2 – Impact of freeze-thaw cycles and of long-term storage

Aim

This second experiment is to assess whether acoustic tubes are appropriate containers to store our compound libraries. Compounds from the five chemical categories have been stored for either long or short periods at both -20 °C and RT and stressed with repeated freeze-thaw cycles. For this experiment, 2 conditions have been tested as follow:

	2020 May							2020 Jun	2020 Jul	2020 Aug	2020 Sept	2020 Oct	2020 Nov	2020 Dec	2020 Jan	2020 Feb	2020 Mar	2020 Apr	
	Week 1				Week 2	Week 3	Week 4												
96 Tube Racks (Temp: +/- -20 °C)	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17	C18	C19	Condition 1
	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12	R13	R14	R15	R16	R17	R18	R19	
Matrix tubes (Temp: +/- -20 °C)	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17	C18	C19	
	R1	R2			R3			R4			R5			R6					
96 Tube Racks (Temp: +/- -20 °C)	C0																			Condition 2
	R1							R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12		
96 Tube Racks (Temp: +21 °C)	C0																			
	R1							R2	R3	R4	R5	R6	R7	R8	R9	R10	R11	R12		

Table 4: Measurements performed over one year for the tubes racks and matrix tubes in both conditions. Representing cycles (C) and readings (R) over the year. C implies that the racks and matrix tubes involved in condition 1, have been stressed with freeze-thaw cycles (E.g: C3 has undergone twice the effects of the freeze / thaw cycles, once when C1 was read and second time when when C2 was read). R means "reading" which denote the action of survey measurement and UPLC analysis (For Matrix tube, only UPCL analysis was applied).

Condition 1: In this condition, the effects of freeze-thaw cycles was tested: The 96 Tube racks and Matrix racks were located in our Liquid Store (LS) at -20 ºC and 60 %RH. The racks were analyzed according to the timeframe detailed in Table 4. Each time that a rack is analyzed (surveyed and QC), all the racks from this condition are moved to the powder store (PS) at 21 ºC and 37 %RH for about 6 h and them moved back to the LS.
Condition 2: In this condition, we were testing the storage of the racks for short/long period in the two different environments: Liquid store and Powder store. Half of the 96 Tube racks are located in the Liquid Store at -20 ºC and 60 %RH and the other half in the powder store at 21 ºC and 37 %RH. The racks are analyzed monthly according to the timeframe detailed in Table 4. For those racks stored at -20 ºC, each time that a rack is analyzed (survey and QC), only the analyzed rack is thawed.

Results

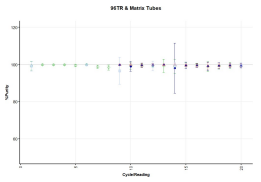


Figure 3: Average of the percentage of purity for all compounds over readings/cycles for all conditions is compared. Condition 1 at -20 °C is represented in color Green, Condition 2 at -20 °C in blue, Condition 2 at RT in Light Red and Condition 1 -20 °C Matrix tubes in Light Blue. Error bars represents the standard deviation for all compounds in one single condition. We have excluded from this plot Rifampicin (see figure 4).

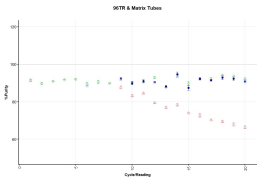


Figure 4: Degradation of the molecule Rifampicin begins after reading 10 (3rd month; Light Red triangles) while stored at RT (Condition2). This plot shows the average of %Purity for Rifampicin over readings under all conditions.

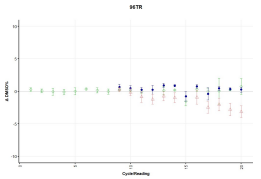


Figure 5: In this graph we compare, the Δ DMSO percentage variability (DMSO% read in one cycle/reading – initial DMSO%) over cycles/readings for 96TR for all conditions. Condition 1 at -20 °C is represented in Green, Condition 2 at -20 °C in blue and Condition 2 at RT in Light Red. Error bars represents the standard deviation of DMSO% for all compounds over the racks. We have excluded from this plot Ofloxacin (See figure 6).

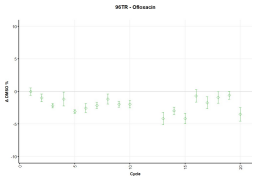


Figure 6: Δ DMSO percentage variability for the molecule of Ofloxacin over time, under condition 1. We observe for this molecule a higher (up to -4.1%) hydration level than any other compounds , and with no apparent trend over time.

Experiment 2 conclusions

- **Condition 1:** No evidence of water uptake or degradation for this condition (see figure 3 & 5, color green) was detected. However we noticed a possible decrease in the % of DMSO for the compound Ofloxacin, through the cycles but in a non-linear way, reaching a maximum decrease of 4.1% from initial reading (see figure 6).
- **Condition 1:** No differences in term of degradation when we store the compounds in Matrix tubes or 96 Tube racks (see fig. 4).
- **Condition 2:** -20ºC: No water uptake or degradation when we store the compounds at -20ºC and no freeze-thaw cycles applied (see figure 3 & 5, color blue).
- **Condition 2:** Room temperature: We observe a small linear decrease of the DMSO% for 96TR stored at room temperature, reaching a minimum of -3.1% after 1 year (see figure 5, color light red).
- **Condition 2:** Room temperature: We observed a clear degradation, over time, for Rifampicin when stored at room temperature in 96TR, reaching a maximum of 20% degradation after 1 year (see figure 4, light red).

Experiment 3 – Sample transfer of highly hydrated solutions

Aim

In this experiment we aimed to study the impact of highly hydrated solution (70% DMSO/water) on compound transfer quantity, using the ECHO, as compared to transfer from a dry solution (100% DMSO).
Compound concentration of hydrated solution were compared with compound concentration from non hydrated solutions. To calculate the final concentration, a calibration curve was performed for each compound at both DMSO percentages (data not shown).

Results

Two compounds were tested for this experiment, 5,5-Diphenylhydantoin and Indomethacin. These compounds have been chosen due to their high solubility in both 100% and 70% of DMSO. Four calibration curves were carried out, one per compound and per % of DMSO, in order to measure final concentration of each compound (for all curves the R^2 was higher than 0.9988). Two independent experiments, and five replicates per points were used in order to determine the calibration curves & the experiments.

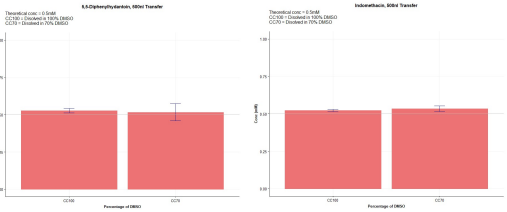


Figure 7: Echo transfer of 600 nl of the molecule 5,5-Diphenylhydantoin dissolved in 100% DMSO (CC100) and 70% DMSO (CC70). Average compound concentration (mM) versus actual transfers in both conditions. Error bars represent the standard deviation of the concentration for each volume dispensed. The grey line represents the theoretical concentration of 0.5 mM intended.

Figure 8: Echo transfer of 600 nl of the molecule Indomethacin dissolved in 100% DMSO (CC100) and 70% DMSO (CC70). Average compound concentration (mM) versus actual transfers in both conditions. Error bars represent the standard deviation of the concentration for each volumes dispensed. The grey line represents the theoretical concentration of 0.5 mM intended.

Experiment 3 conclusions

- No real differences on Echo transfers could be found between compounds transferred from a highly hydrated solution (70%) or a dry 100% DMSO solution (Figures 7 & 8). These results seem promising, but should not be generalized without further testing. It would be reasonable to expect similar results should be obtained with all compounds, but due to the large number of compound chemistry existing and potential change of properties once solubilized in DMSO, it would be possible to assume this could not be the case all the time.

Experiment 4 – Compound integrity during transfer

Aim

The objective of this Experiment was to evaluate the impact of the repeated focused burst of energy required for each transfers on the compound chemical integrity during droplet creation.

Final Vol transferred (uL)	Vol transferred	
1	1 uL	400*2.5nL
10	10 * 1 uL	10*1*400*2.5nL
20	20 * 1 uL	20*1*400*2.5nL
50	50 * 1 uL	50*1*400*2.5nL

Table 5: In order to dispense the final volume we have transferred a maximum of 50 uL in transfers of 1 uL, resulting in 20000 transfers of 2.5 nL.

To perform this experiment, different volumes of each compounds were transferred to random destinations in a plate: 1, 10, 20 and 50 µL. The Echo system can only transfer liquids by repeat transfer of 2.5 nL droplets, meaning that when we dispensing 1 uL, a series of 400 droplets of 2.5 nL had to be dispensed from the source, and so on for larger volumes. The volume range here, was chosen to cover most of the working volume of an acoustic tube (set by the manufacturer at 75 µL).

Compounds at 0.5 mM were prepared and plated in a 96TR. From all compounds, each volumes (1, 10, 20 and 50 µL) were dispensed in triplicate. After each completed transfer, 25 µL of the remaining volume in each source tube was analyzed by UPLC-MS.

Results

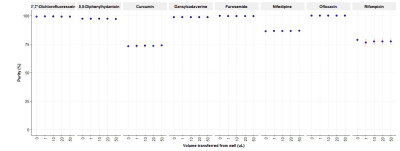


Figure 9: Average purity(%) over the volume transferred in µL is shown for a 96TR initially filled with 75µL of compounds at 0.5mM. Error bars represent the standard deviation of two independent assays for each compound, in triplicates per volume dispensed.

Experiment 4 conclusions

- We have not observed any deterioration in the integrity of the compounds (See figure 9). The % purity measured remained the same for all compounds, independently of volume transferred, hinting that, at least for these selected chemical categories, the sound energy applied to dispense the compounds does not appear strong enough to degrade the molecules.

General Conclusions

- **Equipment-enclosure controlled conditions have shown to enhance performance, in limiting water uptake as well as reducing evaporation when using 96 Tubes Racks, however some evaporation was still observed when using low volume microplates. To minimize DMSO hydration, and ensure good practices, following this work, we have already modified our processes of always using lidded microplates**
- **We have not observed much deterioration regarding compound integrity or compound hydration when compounds are stored under our normal cold storage conditions (-20 ºC), regardless of the number of free-thaw cycles. Only one compound showed higher hydration levels than the rest (-4.1%) which could be explained by its chemical properties**
- **This work showed that we can continue to be confident about acoustic transfers, performed using our Echo, even with respect to mild hydration within our source wells, and regardless of number of transfers required**
- **We are now ready to use the acoustic tubes technology on all our current standard processes of plate preparation and compound distribution for all biological assays**