



## Using the Tecan AC Extraction Plate™ for sample preparation

### A screening study

### The Tecan AC Extraction Plate

The AC Extraction Plate with TICE™ technology (TICE = Tecan Immobilized Coating Extraction) is designed for the extraction of small molecules from biological as well as other liquid samples. Applications include vitamin D quantification in serum, antidepressant detection in urine or screening of painkillers in biological specimen such as serum and plasma. The wells of the plate contain a proprietary coating which absorbs the analyte from an aqueous matrix (“extraction step”) and releases it in the presence of a suitable organic solvent mixture (“elution step”). By varying the characteristics of the liquid phase (e.g., pH, polarity), conditions can be evaluated favoring either the analyte extraction or its elution.

Whether an analyte is suitably extractable by the Tecan AC Extraction Plate is mainly determined by the extraction step. Previous experiments examining the influence of pH and organic content in the solvent indicated that optimum extraction of an analyte into the TICE coating is to be expected for the electrically neutral (i.e. uncharged) form of the target molecule and high water content of the solvent.

For lipophilic molecules, the extraction efficiency was in most cases higher than 60%. Analytes with a more rigid molecular structure (like several annulated rings or an extended steroid skeleton) were found to exhibit a very slow extraction behavior, with an estimated exclusion cutoff for the molecular mass  $\geq 1500$  Da beyond which no extraction is observed.

In this technical note, we have undertaken a screening study examining a range of different analytes with varying functional groups and molecular sizes. The target molecules were either pharmaceuticals, pesticides or simple stereotypical organic molecules. An extraction step was performed in different solvent mixtures composed of water and an organic solvent. To test for charge effects, these solvent mixes were examined at three different pH values. Comparison of the analyte concentration in the supernatant before and after extraction yielded the extraction efficiency.

## Experimental

The various pharmaceutical substances, pesticides and other compounds of interest were subjected to extraction with the AC Extraction Plate. Degree of absorption by the phase was evaluated for a series of LC-MS compatible solvent-water dilutions (methanol, ethanol, isopropanol or acetonitrile) modified with fixed relative amounts of formic acid, ammonium acetate or ammonia, resulting in different pH values (acidic, approximately neutral and basic, respectively).

Extractions were conducted employing a sample volume of 200µl with a 500ng/mL concentration of the respective substance. After addition of the solutions to the wells, the plate was shaken on an orbital shaker at 1100rpm for 10min. The concentration of the substance in the supernatant remaining after extraction was measured by LC-MS/MS.

As a control, the prepared solutions were subjected to LC-MS/MS analysis before extraction. The values for comparison were calculated from the peak area upon ionization. Measurements were performed on each solution in four replicates. No further steps were included to avoid undefined effects on the extraction.

## Results and Discussion

### Analyte compatibility range

The results in Table 1 confirm that a broad range of molecules can be successfully extracted using the Tecan AC Extraction Plate, emphasizing the high suitability of the AC Extraction Plate under various conditions.

The extraction behavior of the AC Extraction Plate is mainly correlated with three known or readily available parameters: (1) the octanol-water partitioning coefficient  $\log P_{ow}$  roughly quantifying the lipophilicity of the molecule, (2) the size of the molecule, (3) the solvent mixture used and (4) its pH value. It is the third and fourth parameters that allow an easy switch between appropriate extraction or elution conditions for many different molecules.

Substance	approx. $\log P_{ow}$	molar mass M	Suitability
Acepromazin	3.5	326 g/mol	good
Albendazol	3.2	265 g/mol	good
Avermectin B1b	5.8	859 g/mol	good
Benzanilid	3.1	197 g/mol	good
Benzophenon	3.4	182 g/mol	good
Benzylbenzoat	3.7	212 g/mol	good
Cholecalciferol	7.1	384 g/mol	good
Clenbuterol	2.3	276 g/mol	not advised
Cloxacillin	2.3	435 g/mol	not advised
Dienestrol	4.8	266 g/mol	good
Efavirenz	4.5	315 g/mol	good
Erythromycin	2.6	733 g/mol	medium
Estradiol	3.8	272 g/mol	good
Flucloxacillin	2.4	453 g/mol	not advised
Ibuprofen	3.8	206 g/mol	good
Indomethacin	3.5	357 g/mol	good
N,N-Dimethylanilin	2.1	121 g/mol	not advised
Naproxen	3.0	230 g/mol	medium
Phenylbutazon	4.1	308 g/mol	good
Pioglitazon	3.3	356 g/mol	good
Thiabendazol	2.3	201 g/mol	not advised
Triphenylamin	5.3	245 g/mol	good

Table 1 Overview of substances used to evaluate the analytes (characteristics) suitable for extraction by the AC Extraction Plate

An extraction efficiency into the coating of more than 90% can usually be expected for sample molecules having a  $\log P_{ow}$  greater than 3.0, provided that a suitable solvent mix and pH is used which favors the neutral form. For molecules with a  $\log P_{ow}$  of 2.5-3.0, an extraction efficiency of more than 60-80% is probable (Fig. 1). However, solubility problems can arise under these conditions since the solvent only contains 0-5% organic.

### logD at suitable pH

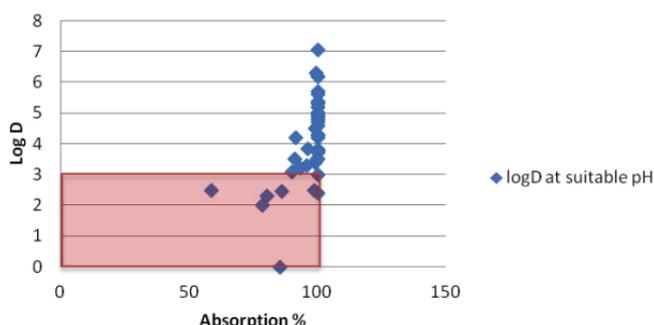


Figure 1 The log D (the weighted average of log POW over all protonation states) is an indicator for the extractability with the AC Extraction Plate. Analyte molecules with a log D value of 3.0 or higher show good absorption into the coating, whereas at least reasonable absorption can be achieved for log D = 2.5-3.0.

### Solvent effects

Varying the organic solvent/water ratio for a pH-inactive compound will result in a sigmoidal curve for the extraction efficiency, starting at a value close to 100% for pure water and decreasing to close to 0% for high organic content. Fig. 2 shows efficiency curves for three different analytes in varying concentrations of acetonitrile while Fig. 3 shows the influence of different solvents on the curve shape and position. The curve shape can be approximated by an inverse exponential function, yielding an extraction efficiency E for any given relative amount X<sub>org</sub> (between 0.0 and 1.0) of organic solvent:

$$E(X_{org})[\%] = 100 \cdot [1 + \exp(-a \cdot X_{org} + b)]^{-1}$$

Exploiting this correlation results in considerable reduction of the experiments needed for finding the optimal extraction or elution conditions. The best results are achieved for data points close to the inflexion point of the curve.

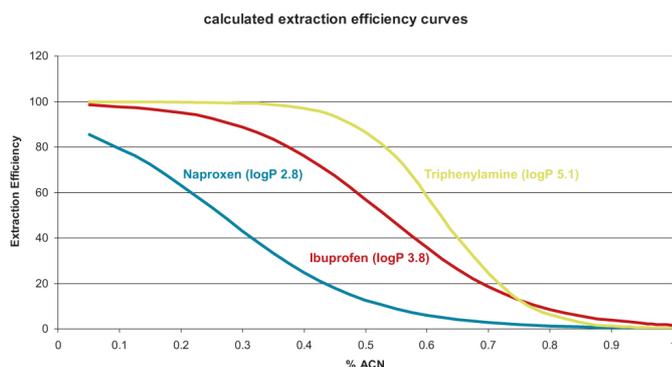


Figure 2 Sigmoidal absorption curves of analytes depending on the acetonitrile concentration in the liquid at neutral pH. Extraction and Elution conditions, as well as defining a suitable wash, can be read from the plot. For a high efficiency of extraction/elution, solvent mixes around the inflexion point of the curve should be avoided whenever possible.

### Molecular size

The molecule size limit for successful extraction with the AC Extraction Plate is estimated to be around 1500 Da, which is mainly attributed to steric hindrance when entering the extraction coating. Thus, macromolecules such as proteins and DNA will not be able to diffuse into the phase. The same, but to a weaker extent, holds true for molecules with a molecular mass below 1500 Da when containing a rigid extended structure, e.g. annulated aromatic rings.

### Effect of pH

For compounds with functional groups that will be protonated/deprotonated depending on the pH value, elution may also be effected by selecting the appropriate pH in a solution with no or very low organic content. It is recommended that the pH value should differ by at least two units from the corresponding pK<sub>a</sub> of the molecule, similar to weak anion exchange chromatography. This ensures that the charged form of the molecule is by far the most abundant form. For further information, please consult the technical note about pH dependency [1].

### Dienestrol

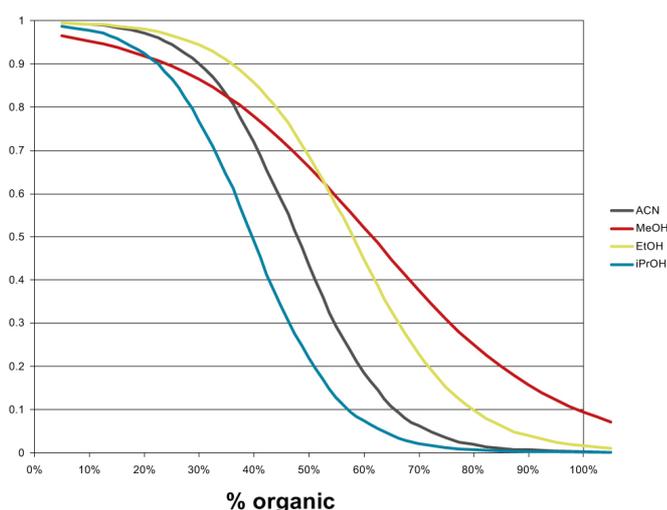


Figure 3 The extraction behavior depends on the type of organic solvent used, here shown for Dienestrol. For methanol, the slope at the inflexion point is significantly less steep than for the other solvents, probably as a consequence of the stronger proticity. Changing the MeOH amount therefore has a much weaker effect onto the extraction efficiency.

## General usage guidelines

It was shown that the Tecan AC Extraction Plate is suitable for an extensive range of analytes. Some general rules can be derived from the experimental data to enable the efficient extraction of a target analyte.

Since the underlying process is a reversible partitioning equilibrium, the results from the extraction step can be directly transferred to find suitable conditions for the elution.

As shown from the experiments conducted here, successful extraction and elution is possible if the following three conditions are met: (1) the log  $P_{OW}$  of the substance is higher than 3.0; (2) the molecular mass of the analyte is below 1500 Da and/or the molecule consists of less than five annulated rings, (3) the analyte molecule has a neutral form. This results that the acidity or basicity of the solution can be chosen such that the neutral form of the target molecule is the most abundant form.

In cases where the log  $P_{OW}$  is 2.5-3.0 the extraction is probably less than 80%, whereas a log  $P_{OW}$  below 2.5 most probably rules out successful extraction.

Optimal extraction conditions typically imply a solvent mixture with a very low organic content (25% or less). For elution from the coating, solvents with high organic content (60-100%) usually give the best results.

Table 2 summarizes the most appropriate conditions for the determination of the extraction/elution parameters of a given analyte.

Parameter	Extraction step	Elution step
log P	> 3.0 (> 2.5)	
molecular mass/size	M < 1500 Da / less than 5 rings	
electronic properties	molecule must have a neutral form	
% organic solvent	0-25%	60-100%/0-5%*
charge state	neutral	neutral/charged*

*Table 2 Favorable conditions for the determination of extraction/elution parameters for a given analyte. Please note that the entries marked with an asterisk apply to molecules which have a charge during the elution step.*

Finally, for the appropriate adaptation of sample preparation methods which were previously performed with SPE, it must be kept in mind that the effect of hydrogen bonding properties and polarity of an organic solvent on the absorption/desorption equilibrium could differ significantly when using the AC Extraction Plate.

## References

1. Technical Note: Efficiency of extraction: the influence of pH. <http://www.tecan.com/consumables>.

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