



## Technical Note

***Sample preparation by solid-phase extraction  
for the analysis of (very) polar molecules in salty aqueous samples  
with supercritical fluid chromatography (SFC) – UV spectroscopy***

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### **Abstract**

In this study, small, polar and mostly aromatic organic molecules are effectively extracted from a very salty aqueous sample using (novel) solid-phase extraction (SPE) materials and subsequently analyzed by (polar column) supercritical fluid chromatography (SFC) in combination with UV detection.

The utilized solutions mimicked industrial wastewaters and contained the exemplary phenolic compounds 2-aminobenzoic acid, vanillic acid, syringic acid, 3-hydroxybenzoic acid, non-aromatic maleic acid, 3,5-dihydroxybenzoic acid, and 2,5-dihydroxybenzoic acid as well as respectively 0, 1, 5, 10, 15, 20 or 25% sodium chloride.

The SPE materials Strata C18-E, Strata CN, Strata-X 33  $\mu\text{m}$  and Strata-X Pro was kindly provided by our partner Phenomenex LTD (Aschaffenburg, Germany) and were tested in the cartridge dimensions of 200mg / 3mL and 500mg / 6mL. The analysis and the determination of recovery rates of organic compounds were performed with polar SFC-UV. The salt content of the SPE eluates was determined by colorimetric testing for chloride. Strata X-33 and Strata X-Pro phases had the highest recoveries of organic molecules by reducing the salt content to less than 0.1 g/L in both cartridge dimensions. Thereby, the salt content does not influence the recovery rate of polar and very polar compounds for these phases. Consequently, this sample preparation strategy seems to be a very suitable concept for the desalting of aqueous samples without a relevant loss of (the applied) organic compounds. Forthcoming experiments will give an impression how valid this SPE cartridges will be useable in mass spectrometric non-target screening workflows.



## Introduction

Non-target screening (NTS) is a comprehensive analytical strategy which offers to gain a broad perspective on complex samples containing organic molecules. Although, the analytical strategy behind NTS is suitable for the detection of a wide range of different organic compounds, the sample composition can be a challenge for the analysis. In that consequence polarity-extended chromatographic techniques like RPLC-HILIC and SFC are often used for the separation of samples with non-polar, polar and/or very polar compounds [1,2,3]. The monitoring of latter molecule class is highly challenging if it comes to samples with high contents of inorganic salts due to negative effects in chromatography and mass spectrometric detection. Thus, techniques for sample preparations are urgently needed to remove salts from the solution without loss of (very) polar organic molecules.

As part of the research project RIKovery (supported by the German Federal Ministry of Education and Research), samples with sodium chloride content up to 25% shall be analyzed by NTS. Some of the samples are expected to contain phenolic compounds and will be treated with advanced oxidation processes. As a consequence, the presence of polar and very polar molecules in the samples is very likely. The contained salts are not suitable for direct chromatographic separation and mass spectrometric analysis. Thus, a sample pre-treatment step is required. It should be capable of removing inorganic salts like sodium chloride but also show high recovery rates for polar and very polar organic compounds.

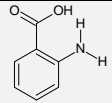
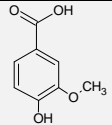
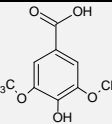
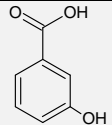
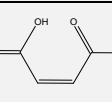
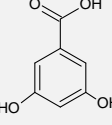
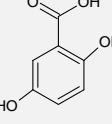
In this study, the applicability of solid phase extraction (SPE) for the removal of sodium chloride from aqueous samples was investigated. Therefore, four different SPE phases were tested and SFC-UV was applied for polar molecule analysis. The valuable knowledge of useable SPE material will be the basis for following NTS studies.



## Material and methods

### Chemicals and solutions

Table 1: Applied compounds with study relevant information

Analytes	Retention time [min]	log D (pH 7)	InChiKey <sup>1</sup>	Structure <sup>2</sup>
<b>2-aminobenzoic acid (2-AB)</b>	4.5	-0.8	RWZYAGGXGHYGMB-UHFFFAOYSA-N	
<b>Vanillic acid (VAN)</b>	4.7	-1.6	WKOLLVMJNQIZCI-UHFFFAOYSA-N	
<b>Syringic acid (SYR)</b>	4.9	-1.9	JMSVCTWVEWCHDZ-UHFFFAOYSA-N	
<b>3-hydroxybenzoic acid (3-HB)</b>	5.7	-1.7	IJFXRHURBJZNAO-UHFFFAOYSA-N	
<b>Maleic acid (Mal)</b>	6.5	-4.8	VZCYOOQTPOCHFL-UPHRSURJSA-N	
<b>3,5-dihydroxybenzoic acid (3,5-DHB)</b>	7.9	-2.1	UYEMGAFJOZZIFP-UHFFFAOYSA-N	
<b>2,5-dihydroxybenzoic acid (2,5-DHB)</b>	9.0	-1.8	WXTMDXOMEHJXQO-UHFFFAOYSA-N	

<sup>1</sup> <https://water.for-ident.org> and <https://pubchem.ncbi.nlm.nih.gov/> <sup>2</sup> by ACD/ChemSketch (Freeware)

LC-MS grade methanol (MeOH) was obtained from Honeywell/Riedel de Haen (Seelze, Germany). Water (LC-MS grade ammonium acetate and sodium chloride) were purchased from Merck KGaA (Darmstadt, Germany). Standard compounds 2-aminobenzoic acid (2-AB), vanillic acid (VAN), syringic acid (SYR), 3-hydroxybenzoic acid (3-HB), maleic acid (Mal), 3,5-dihydroxybenzoic acid (3,5-DHB), and



2,5-dihydroxybenzoic acid (2,5-DHB) were purchased from Alfa Aesar (Thermo Fisher Scientific, Karlsruhe, Germany), Fluka (Buchs, Switzerland) and Merck KGaA (Darmstadt, Germany).

The test solutions contained 0, 1, 5, 10, 15, 20 and 25% sodium chloride, respectively and 100 mg/L of each of the seven (very) polar organic compounds. Physicochemical details and structure can be seen in Table 1.

#### Solid Phase Extraction – Materials and Procedure

The study included SPE phases with different basic retention modes. These were Strata C18-E, Strata CN, Strata-X 33  $\mu$ m and Strata-X Pro (all Phenomenex LTD, Aschaffenburg, Germany). All phases were tested in the dimensions 200 mg/3mL and 500 mg/6 mL, respectively. The procedure of both dimensions is presented step by step in Table 2.

Table 2: Solid-phase extraction procedure in two cartridge dimensions

SPE protocol	200 mg/3 mL	500 mg/6 mL
Conditioning	3 mL methanol	6 mL methanol
Equilibration	3 mL water	6 mL water
Loading	3 mL sample	6 mL sample
Washing	3 x 3 mL water	3 x 6 mL water
Drying	2.5 min vacuum	5 min vacuum
Elution	2 x 1.5 mL methanol/water 80/20 (v%/v%)	2 x 3 mL methanol/water 80/20 (v%/v%)



### Solid phase extraction instrumentation

The SPE procedure was performed with a vacuum manifold (kindly provided by Phenomenex LTD Aschaffenburg, Germany) for 12 SPE cartridges (Figure 1). Vacuum was provided by a vacuum pump (Vacuubrand, Wertheim, Germany). During all steps of the SPE procedure the pressure was adjusted to ensure a maximum flow through the cartridges of approximately 1 drop per second. In most cases this was achieved with -10 to -20 kPa. For the drying step a vacuum of -50 kPa was applied.



Figure 1: Solid-Phase Extraction Chamber with placed cartridges

### Colorimetric Chloride Test

The sodium chloride content in SPE treated samples was determined using a colorimetric test based on mercury (II) thiocyanate and its reaction with chloride (Merck, Darmstadt, Germany). The presence of chloride leads to the formation of iron(III) thiocyanate, which results in a red color. The chloride concentration is determined semi-quantitatively via visual comparison of the color of the reaction



solution with a reference (color card, etc.). The influence of methanol on the reaction was evaluated measuring different sodium chloride concentrations in methanol/water (80/20 (v%/v%)). For the determination of sodium chloride concentrations in SPE eluates, the solutions were compared visually with a 0.1 g/L sodium chloride solution in methanol/water (80/20 (v%/v%)).

#### Supercritical Fluid Chromatography – UV detection

For the recovery of the added polar and very polar compounds, SPE treated samples were analyzed by supercritical fluid chromatography (SFC) with UV detection. The analytical SFC system (Agilent Technologies, Waldbronn, Germany) consisted of a degasser, a binary pump with solvent selection valve, an autosampler with 10 µL injection loop, a thermostatically controlled column compartment, a diode array detector and a backpressure regulator unit. Data acquisition and evaluation were done with Agilent OpenLab ChemStation.

The separation was performed, using a CO<sub>2</sub> –MeOH (20mM NH<sub>4</sub>Ac containing) gradient from 5 to 60 % within 6 minutes (with 1 min isocratic 5% B at the beginning) using a Eurosphere II HILIC column (150 x 3 mm, 3 µm) from KNAUER (Berlin, Germany). The flow rate was 2.0 mL/min, backpressure was set to 130 bar and column temperature was held constant at 40°C. The UV absorption at 210 nm was recorded

10µL of standard solutions were injected in triplicate and each separated under these conditions. The calibration solutions were injected in the concentration levels from 5mg/L to 150mg/L, respectively. The recovery of compounds after the desalting step was recalculated using the external calibration curves.



## Results and discussion

The seven compounds (with logD values in the range from -0.8 to -4.8; details see Table 1) could be separated in a good performance with high k values and baseline separation (see Figure 2) using (polar column) SFC. Consequently, RTs were observed with relative standard deviations in the calibration measurements below 0.7% over the total study period. The compounds in real samples (subsequent to the desalting step) behaved the same. Further, the compounds could be monitored with robust UV detection (at 210 nm wavelength). The recovery studies for the applied compounds were performed in a calibration range like presented in Figure 3. Thereby the relative standard deviations of the signal area were (in lowest concentration) at 1% (Mal, 3,5 DHB), 3% (2-AB, 2,5 DHB), 6% (3-HB), or 9% (VAN, SYR) and at the higher concentrations always below 4%, however mostly about and below 1% (i.e., 89% of the peaks). Further, the linearity of the six UV values of each compound calibration was given with R values better than 0.999 (only 2-AB with 0.998). Thus, the analysis and calibration were robust and reproducible and further used for the recovery study.

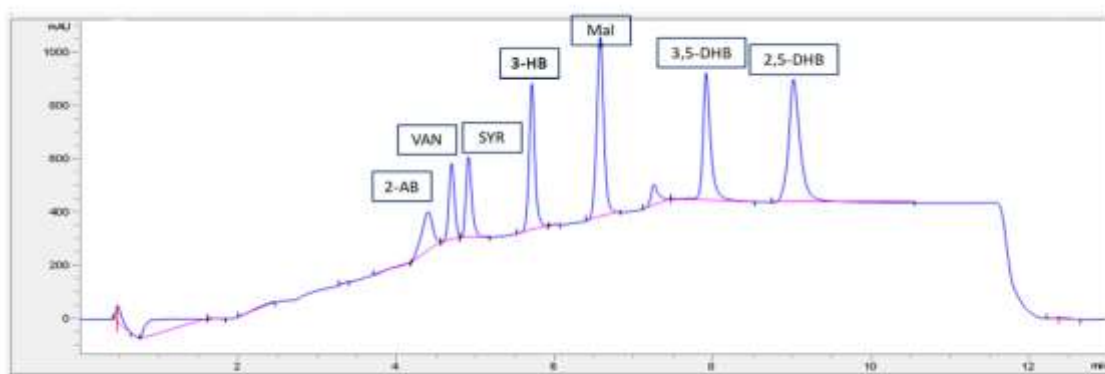


Figure 2: UV-Chromatogram (at 210nm absorption wavelength) including seven compounds observed by SFC-UV analysis.

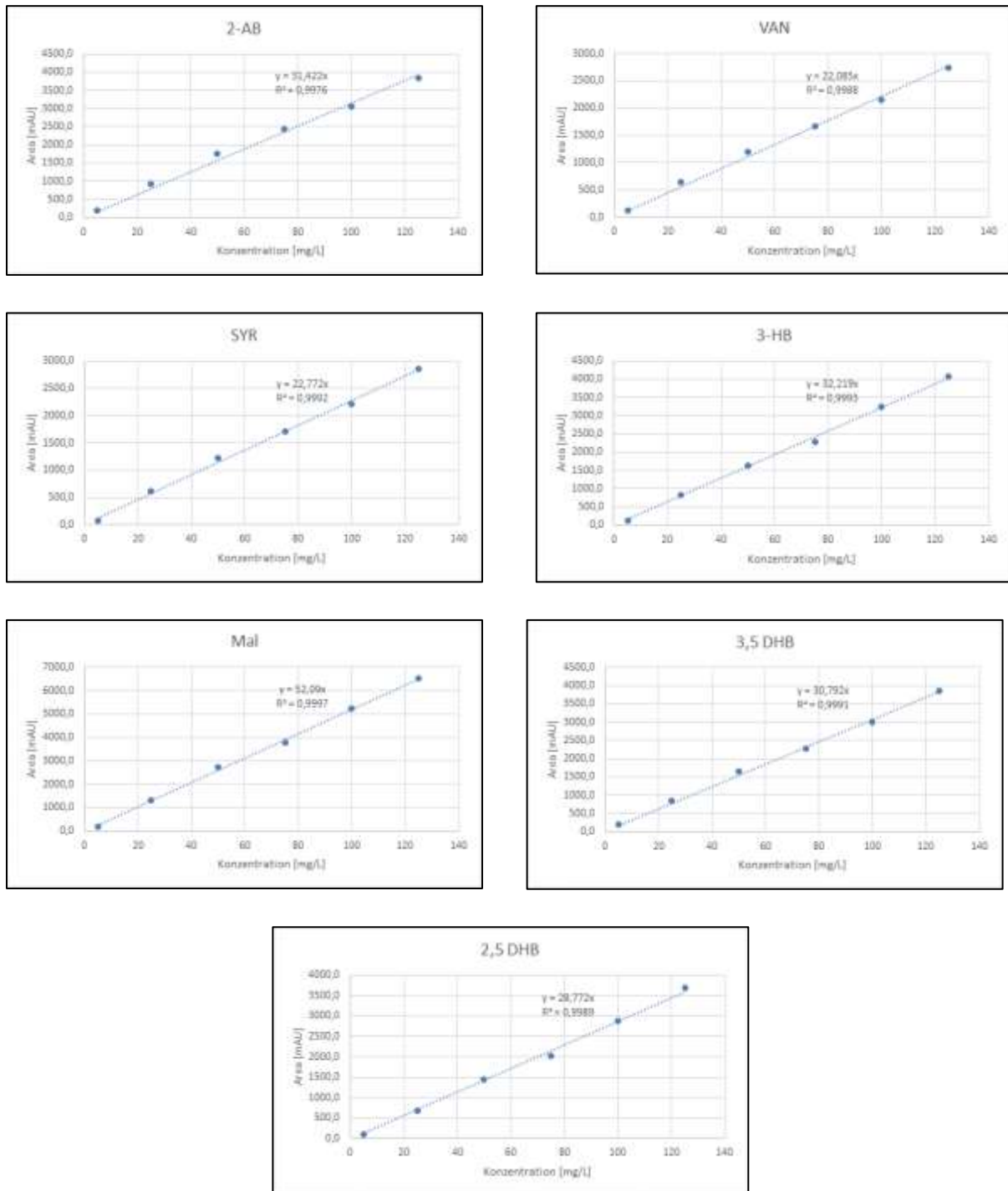


Figure 3: Calibration of the seven applied reference standards on SFC-UV (at 210nm)





In a further step the samples with the reference standards and various concentrations of sodium chloride, respectively, were desalted by the SPE procedure. The resulting eluates were analysed with the validated quantitation method for recovery rates if the salt content was below 0.1 g/L. This was determined by comparing the results of the colorimetric assay of the eluates with a 0.1 g/L solution (Figure 4)

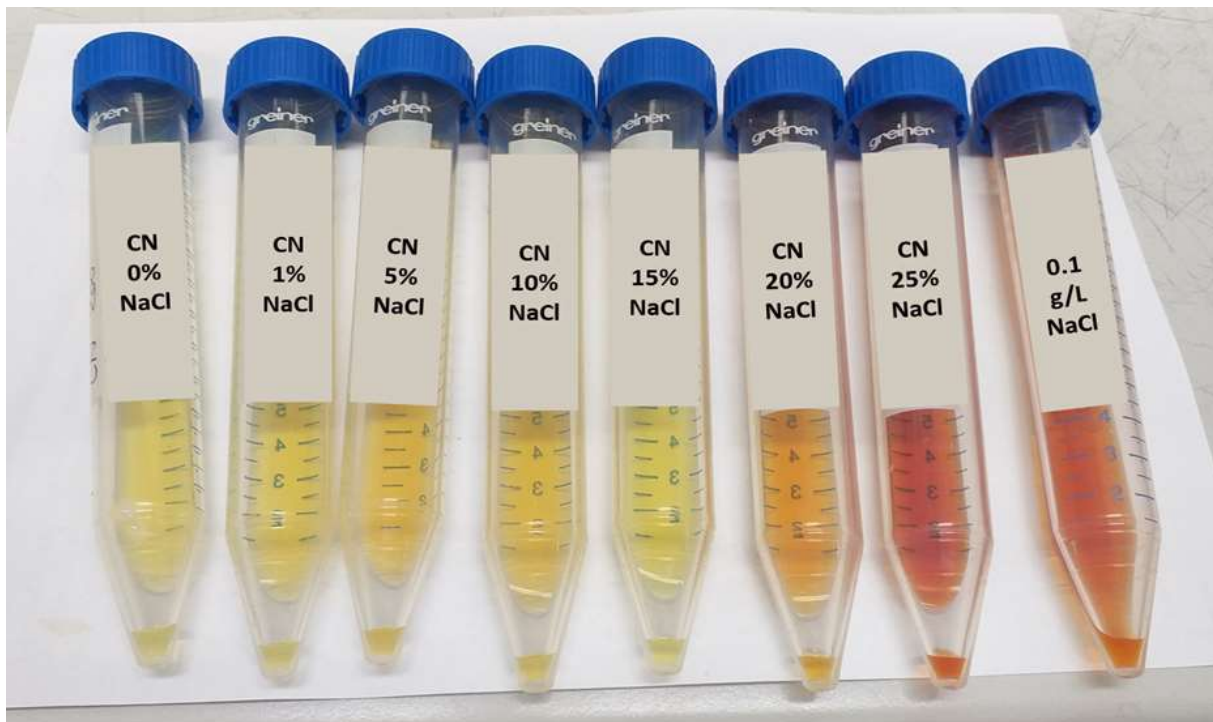


Figure 4: Results of the colorimetric assay on the eluates of different concentrated sodium chloride solutions on the cyano SPE phase, compared to a 0.1 g/L solution (right side).

The desalting procedure described above was applied to the four SPE phases. They were all capable of reducing the salt content below 0.1 g/L (see Table 3). Only, the Strata CN material (in both cartridge dimensions) could not desalt the 25% NaCl containing solution below the effectively needed value. Thus, this solution was not considered in the study further on. All details of the salt contents in the SPE eluates can be found in Table 3.

Table 3: Sodium contents of the solid-phase extraction eluates for both cartridge dimensions after the cleaning procedure as shown in Table 2

	0%	1%	5%	10%	15%	20%	25%
Strata C18-E 200 mg/3 mL	-	< 0.01	< 0.01	< 0.01	< 0.1	< 0.1	< 0.1
Strata CN 200 mg/3 mL	-	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	> 0.1
Strata X 33 µm 200 mg/3 mL	-	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Strata-X Pro 200 mg/3 mL	-	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Strata C18-E 500 mg/6 mL	-	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Strata CN 500 mg/6 mL	-	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	> 0.1
Strata X 33 µm 500 mg/6 mL	-	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
Strata-X Pro 500 mg/6 mL	-	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1

The recoveries of studied analytes in desalted eluates were determined by SFC-UV analyses as described above. Figure 5 and 6 represent the detailed recoveries (with standard deviation by triplicates) of all organic molecules in several salty solutions in both SPE cartridge dimensions. Thereby, Strata X-33 and Strata X-Pro reflect excellent recovery rates among investigated phases (whereas CN could not retain any organic compound and C18 only significantly reduced). Both Strata X materials reached recoveries between 60% and 120% for all phenolic compounds and even for maleic acid up to 50%.





Figure 5: Recovery rates of studied compounds in the eluates for both cartridge dimensions.



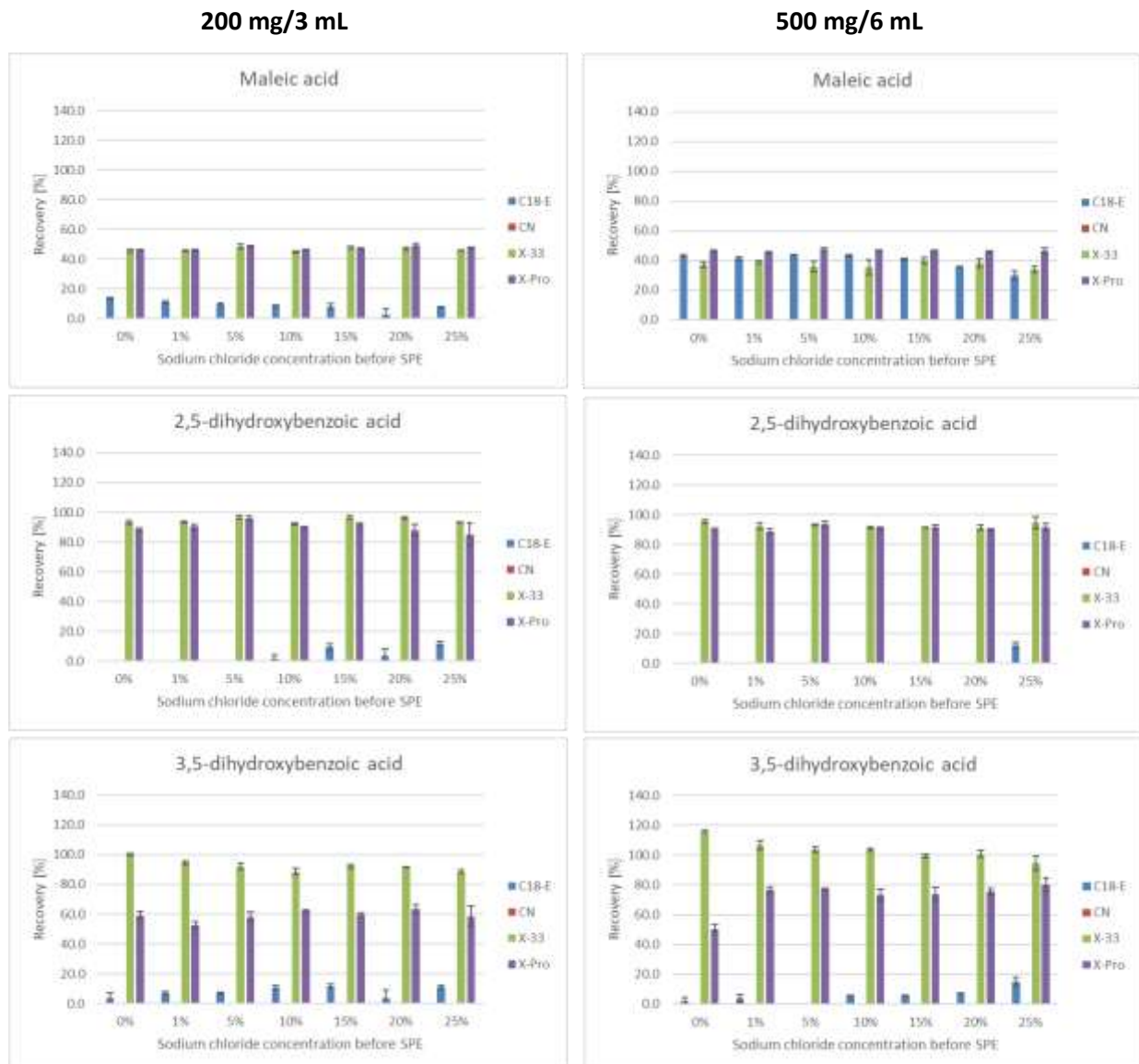


Figure 6: Recovery rates of studied compounds in the eluates for both cartridge dimensions (continued).

Overall, Strata X-33 provides a significantly better recovery than Strata-X Pro in the cartridge dimension 200mg / 3mL. Especially for the phenolic compounds, the observed recoveries after using Strata X-33 in 200mg / 3mL were more closely to 100 %, whereas Strata X-Pro in 200mg / 3mL and both phases in 500mg / 5mL resulted sometimes in significant overdeterminations.



## Conclusion

Solid Phase Extraction with Strata X-33 and Strata X-Pro materials has been shown to be a very suitable concept for the desalting of aqueous samples. Overall, the salt content does not influence the recovery rate of polar and very polar compounds for these phases. Especially the SPE material Strata X-33 in the cartridge dimension 200mg / 3mL seems to be best fitting to extract small, slightly acidic and polar organic compounds out of salty solutions.

In next studies the phases are applied for the extraction of further polar organic compounds to handle salty solutions also in today's non-target screening solutions.

## Acknowledgements

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