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Deliverable Number:	D3.1
Work package number:	WP3
Deliverable title	DETECTION AND CHARACTERIZATION PROTOCOLS OF SELECTED EC'S
Type	Report
Dissemination Level	Public
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Scheduled delivery date	20 April 2017
Actual / forecast delivery date	1 May 2017

Deliverable summary

This deliverable summarizes detection and characterization protocols of several emerging contaminants (ECs): gold, silver and copper nanoparticles, and the pharmaceutically active substances Enalapril, Oxaliplatin and Fluorouracil (5-FU). The report includes detailed sample preparation for analysis and analytical methods and parameters used for characterization. Overall, we provide here detailed protocols for the synthesis, characterization and detection of these compounds in aqueous solutions, in environmentally-relevant concentrations. These methods will be used as the foundation for the next deliverables that are related to the study of transport, fate and risk assessment of these compounds in the environment.

D3.1

Detection and characterization protocols of selected emerging contaminants (EC's)

1. Introduction

This deliverable summarizes detection and characterization protocols of several emerging contaminants (ECs): gold, silver and copper nanoparticles, and the pharmaceutically active substances Enalapril, Oxaliplatin and Fluorouracil (5-FU). The report includes detailed sample preparation for analysis and analytical methods and parameters used for characterization. Overall, we provide here detailed protocols for the synthesis, characterization and detection of these compounds in aqueous solutions, in environmentally-relevant concentrations. These methods will be used as the foundation for the next deliverables that are related to the study of transport, fate and risk assessment of these compounds in the environment.

2. Selected ECs - Synthesis and properties

2.1 Synthesis of engineered nanoparticles (ENPs)

2.1.1. Gold nanoparticles - Au-NP suspensions were synthesized using the citrate reduction method described previously by Frens [1]. Briefly: 0.5 mM of $HAuCl_4 \cdot 3H_2O$ (Sigma) were boiled with 1.7 mM trisodium citrate (Sigma) in 500 mL deionized water (18.2 MΩ); after the suspension turned red it was stirred vigorously and kept boiling for 15 min. For the Ag-NP suspension, 10 mL of 0.1 M $AgNO_3$ (Sigma) were added to boiling citrate solution (990 mL, 7 mM, pH adjusted to 11.5 with (NaOH) under vigorous stirring for 15 min.

2.1.2 Silver nanoparticles - Ag-NP suspensions were synthesized using the citrate reduction method described previously by Dong et al. [2]. Briefly: 10 mL of 0.1 M $AgNO_3$ (sigma) was added to 990 mL of boiling 7 mM trisodium citrate solution (pH adjusted to around 11.0 with 1N NaOH) under vigorous stirring for 15 min. Color of the suspension changes from colorless to yellow and then turbid blackish blue. Volume may be adjusted at the end to 1000 mL which makes AgNP suspension of concentration 108 ppm.

2.1.3 Copper nanoparticles - Cu-NP: Stock solution of 1.6 mM PEI polyethylenimine (PEI; $H(NHCH_2CH_2)_nNH_2$, branched, MW = 25,000 Da; Sigma-Aldrich) in DI water is prepared. A volume of 7 mL of the PEI stock solution was then mixed for 5 min with 10 mL of 250 mmol/L $Cu(NO_3)_2 \bullet 3H_2O$ solution and a complementary aliquot of DI water to achieve total volume of 40 mL. During this stage, the solution color was dark blue due to formation of Cu-PEI complexes. Subsequently, addition of 10 mL of 0.5 mol/L $NaBH_4$ to the solution reduced the soluble copper to elemental copper, followed by formation of Cu-NPs. The 50 mL Cu-NP suspension is stirred (~350 rpm) for 1 h and then dialyzed for 24 h (Cellu Sep: 3500 MWCO, Membrane Filtration Products, Inc., TX, USA) in glass

beakers filled with 950 mL DI water. The concentration of CuNP suspension before dialysis was 3177 ppm.

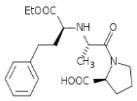
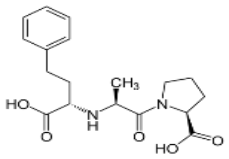
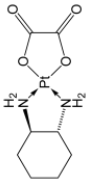
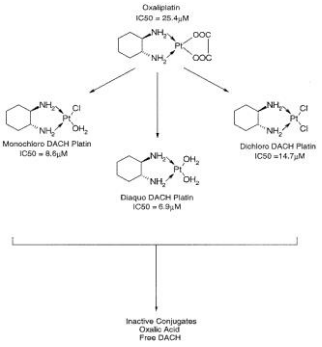
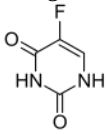
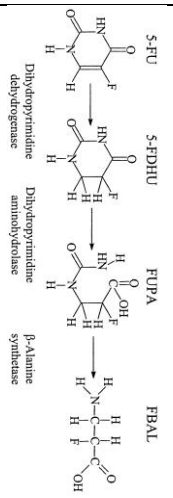
2.2 Selected pharmaceutically active substances

2.2.1 Enalapril (purchased as the salt enalapril maleate) is a medication used to treat high blood pressure, several types of heart failure and diabetic nephropathy (see Table 1 for chemical properties). Merck introduced enalapril to the market in 1981; it became Merck's first billion-dollar selling drug in 1988. The patent expired in 2000, opening the way for generics.

2.2.2 Oxaliplatin is a platinum-based chemotherapy pharmaceutical, typically used to treat colorectal cancer (see Table 1 for chemical properties). Oxaliplatin was patented in 1976 at Nagoya City University by Professor Yoshinori Kidani and approved for medical use in 1996. It is on the World Health Organization's List of Essential Medicines, the most effective and safe medicines needed in a health system.

2.2.3 Fluorouracil (5-FU) is a medication used to treat cancer (colon cancer, esophageal cancer, stomach cancer, pancreatic cancer, breast cancer, cervical cancer and basal cell carcinoma). Fluorouracil was patented in 1956 and came into medical use in 1962. It is on the World Health Organization's List of Essential Medicines, the most effective and safe medicines needed in a health system. The basic chemical properties of 5-FU are given in Table 1.

Table 1. The properties of enalapril, oxaliplatin and fluorouracil.

Compound + Mw	**Formula**	**K_{OW}**	**pK_a at 25°C**	**Known Metabolites *In Vivo***	**Detected Concentrations** ***
Enalapril 376.44 gr/mol 	$C_{20}H_{28}N_2O_5$	0.16 [3]	2.97 (carboxyl) 5.35 (amine)	 Enalaprilat	WWTP influent: 201-369 ppt WWTP effluent: 239 ppt (Canada) [1]
Oxaliplatin 395.27 gr/mol 	$C_8H_{12}N_2O_4Pt$	0.89 [4]	7.23 [5]	 [6]	<150 ppt [7]
Fluorouracil 130 g/mol 	$C_4H_3FN_2O_2$	0.13 [8]	8.02 [9]	 [10]	<100ppt [11]

3. Characterization of the Selected ECs

3.1 Characterization of ENPs

Transmission electron microscopy (TEM) imaging was used to determine ENP shape and size, performed with a Philips CM-120 instrument operating at 120kV, equipped with a CCD camera (2k×2k Gatan Ultrascan 1000). TEM samples were prepared by placing a 5 μL drop on ecollodion/carbon-coated 400 mesh Cu grid and blotting after one minute. An EDAX energy dispersive spectrometry (EDS) system was used to perform elementary analysis. TEM imaging and EDAX analysis for AgNPs and AuNPs are given in Figure 1. EM size, DLS measurements and ζ potential of ENPs under different chemical conditions are summarized in Table 2

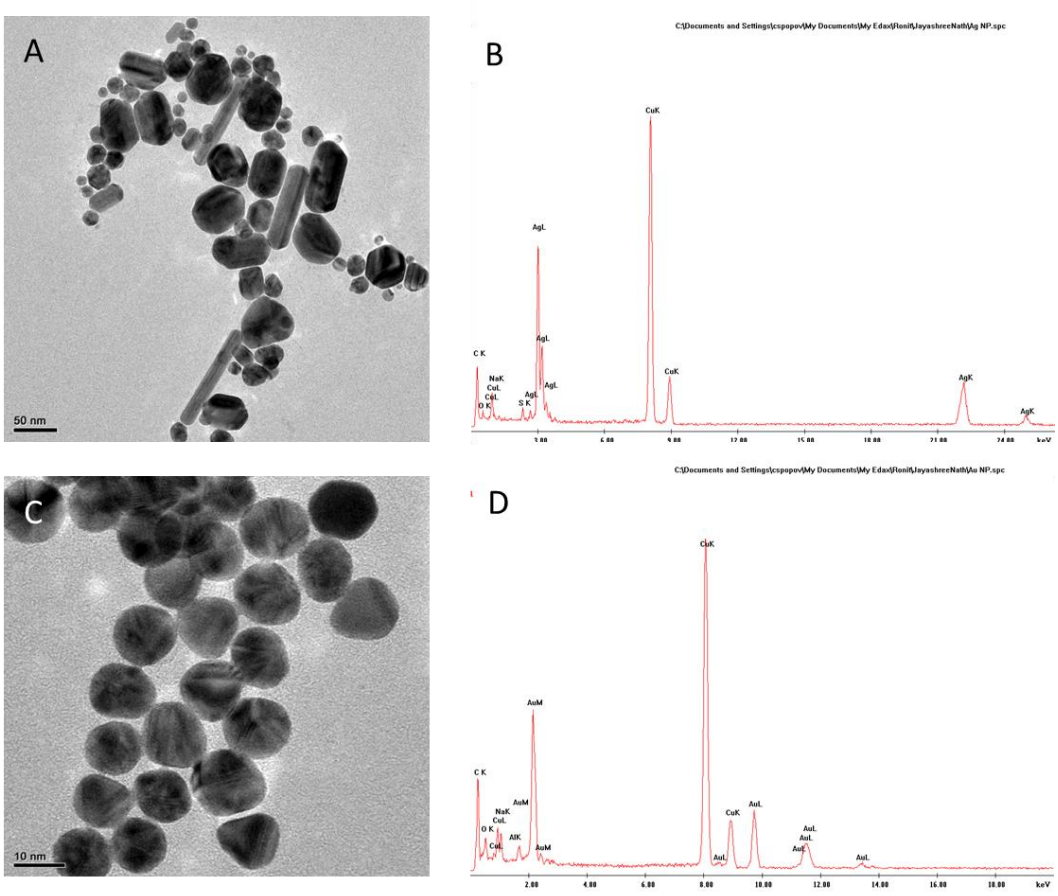


Figure 1. Transmission electron micrograph (A and C) and EDS analysis (B and D) of silver and gold nanoparticles, respectively.

Table 2. ENP size (diameter) characterization and zeta (ζ) potential.

ENP	TEM [nm]		DLS [nm]	Zeta [mV]
Ag-NPs	Average	51.7	79 (5.3)	-55.5 (1.9)
	Standard deviation	16		
	Median	53.5		
	Sample size	85		
Ag-NPs + humic acid 5 mg L^{-1}			79 (0.8)	-52.7 (2)
Ag + $CaCl_2$ 15 mg L^{-1} (10 min)			77 (2.5)	-42.3 (1.3)
Ag + $CaCl_2$ 300 mg L^{-1} (10 min)			138 (15)	-22.7 (0.8)
Ag_2S-NPs	Average	37.2	84 (2.4)	-51.3 (1.7)
	Standard deviation	14.5		
	Median	37.5		
	Sample size	64		
Au-NPs	Average	21.2	49 (0.4)	-43.8 (1)
	Standard deviation	6.4		
	Median	19		
	Sample size	208		
Au-NPs + humic acid 5 mg L^{-1}			50 (0.7)	-41.2 (0.9)
ZnO-NPs	Average	32.4	70 (5.6)	10.9 (1.4)
	Standard deviation	10.8		
	Median	29.6		
	Sample size	204		
ZnO-NPs + humic acid 5 mg L^{-1}			280 (15)	-23.7 (0.7)
ZnO-NPs + humic acid 50 mg L^{-1}			151 (14)	-26.8 (2.9)

3.2 ENP quantification

ENP concentrations were measured by inductively coupled plasma - mass spectrometry (ICP-MS) (Agilent 7700), and based on a calibration curve of ENP suspensions used in the same experiment and prepared by the same protocols. Results are given as ratio of outlet to inlet concentrations (C/C_0). Sample preparation procedures to determine BTCs and RPs were as follows: for aqueous phase samples, aliquots from the collected fractions were diluted with acid and then digested for 48 h to allow ENP dissolution. For solid phase samples, 20 mL acid was added to each sand sample, followed by 48 h shaking. Then the sand was separated and the supernatant was analyzed. Extraction efficacy (generally >90%) was determined for each experiment separately and accounted for in yield calculations. For Ag-NPs and ZnO-NPs, acid concentrations were 3% nitric acid. For Au-NPs, a mixture of nitric acid (1%) and hydrochloric acid (3%) was applied. When silver chloride precipitates were expected, the samples were mixed with 5% ammonium hydroxide.

3.3 Detection protocols for the selected pharmaceutically active substances

3.3.1 Enalapril and its main metabolite enalaprilat

were detected by Liquid Chromatography-Mass Spectrometer (Waters, 1525 binary HPLC pumps, 515 HPLC pumps, Quattro microTM APT ESCIiTM Micromass, 2777c Sample manager). The

dissolved species pass through an on-line solid-phase extraction (SPE) column, are separated in the chromatographic column, then released, ionized and detected according to their masses and the pre-determined MS detection method. The Electro Spray Ionization (ESI) MS detector produces little fragmentation, and tandem mass spectrometry (MS^2) enables detecting a pharmaceutical and some of its known fragments simultaneously. The instrument allows detection of the known, pre-determined transformation products as well as the unknown transformation products.

Enalapril samples were acidified by adding formic acid to a final concentration of 0.5% to ensure full enalapril protonation and enhance the detection in the positive MS mode. In addition, an isotopically labeled enalapril-d5 maleate was used as an internal standard. The LC method is specified in Table 3. The MS^2 method monitors two daughter ions: 377.20 → 91.20 m/z, 377.20 →234.40 m/z.

Data were processed by MassLynx software (V4.1 2005). Concentrations were calculated by integrating the area below the mass peaks, according to a calibration curve. Dilution corrections were applied to the calculated concentrations. Steady, reproducible signals were acquired for concentrations as low as 0.1 ppb in a 0.5 mL injection (in DDW; Fig. 2 and 3). The low detection limit, the on-line SPE, and the small sample volume required for a stable signal enable us to work with environmentally-relevant concentrations of pharmaceuticals and small volumes of inlet solutions, thus saving time and sample preparation steps that induce uncertainties and errors.

Table 3. The LC method.

Loading Phase*				Elution Phase**			
Time [min.]	Flow	%A	%B	Time [min.]	Flow	%A	%B
Initial	4.00	100.0	0.0	Initial	0.60	100.0	0.0
0.20	4.00	100.0	0.0	3.00	0.60	100.0	0.0
4.00	4.00	100.0	0.0	3.10	0.60	60.0	40.0
4.10	0.20	100.0	0.0	6.00	0.60	0.0	100.0
12.00	4.00	100.0	0.0	11.00	0.60	0.0	100.0
12.50	4.00	100.0	0.0	11.10	0.60	100.0	0.0
15.00	4.00	100.0	0.0	12.50	0.60	100.0	0.0
				15.00	0.60	100.0	0.0

* A- 97% H_2O + 3% acetonitrile + 0.1% formic acid; B- methanol.

** B- 95% water + 5% acetonitrile +0.1% formic acid.

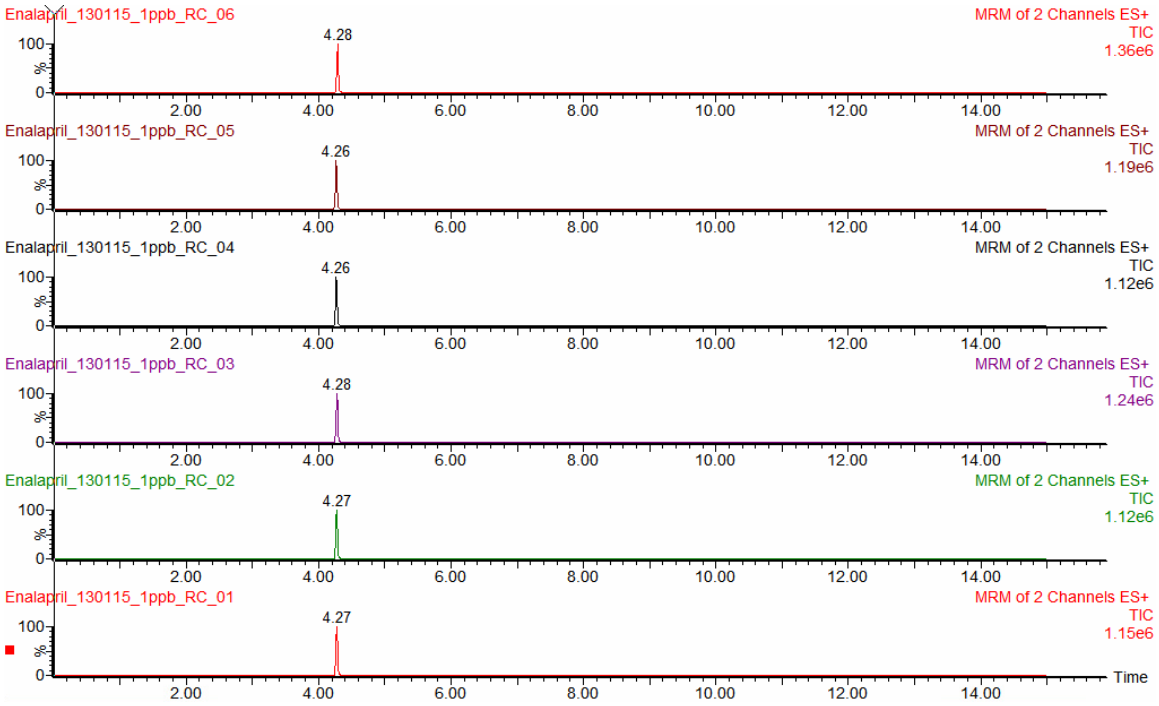


Figure 2. Repeatability of a 1 ppb signal of Enalapril in DDW, 1% formic acid.

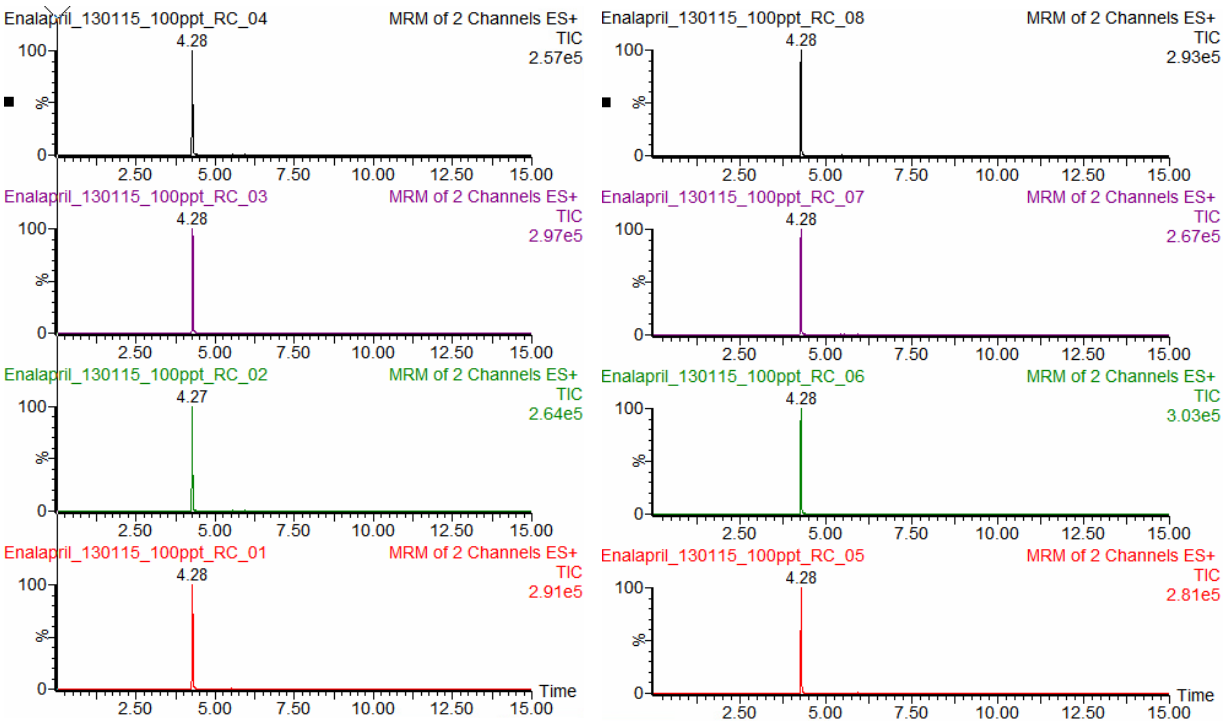


Figure 3. Repeatability of a 100 ppt signal of Enalapril in DDW, 1% formic acid.

3.3.2 Oxaliplatin species were detected as Pt by Inductively Coupled Plasma Mass Spectrometer (ICP-MS) Agilent 7700s. The detected isotopes were ^{194}Pt, ^{195}Pt. Oxaliplatin samples were filtered through a 0.45 µm syringe filter, and acidified with nitric acid to a final acid concentration of 2% to enhance species ionization, and stored in 4˚C until analysis. A solution of 1 ppm Gd in 1% nitric acid was used as an instrumental internal standard to monitor the stability of ionization efficiency in time. The measurement was performed in a collision mode with $He_{(g)}$ to minimize interferences, at 5 mL/min. The extraction lens potential was set to -185 V. The dwell time per isotope was 0.10 s. A quick scan of 20 additional elements was performed without $He_{(g)}$, for a qualitative determination of major element composition of the solution.

Sample intake lasted 30 s, at pump speed of 0.3 rps. Between samples, the probe was rinsed with a 3% nitric acid solution for 40 s, followed by a 1% nitric acid solution for 20 s (pump speed: 0.3 rps). Every 20 samples, a calibration standard of either 10 ppb or 20 ppb was repeated to check for carryover and detection stability.

Data were processed by the MassHunter 4.1 software, version C.01.01, 2015 (Fig. 4). Results recorded in counts/second were converted to ppb according to a calibration curve. Internal standard drift and dilution corrections were applied to the detected Pt concentrations.

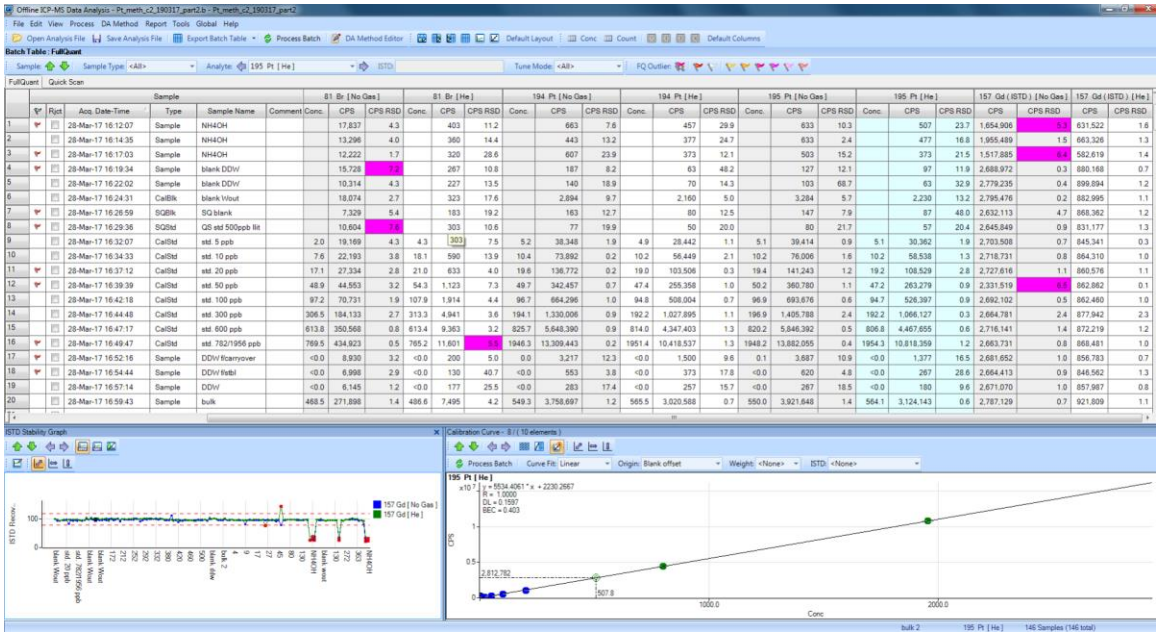


Figure 4. Data analysis by MassHunter.

3.3.3 Fluorouracil (5-FU) analytical protocol. The analysis of 5-FU was done by HPLC. For 5-FU measurement: Waters 1525 binary HPLC pump was used with a Symmetry C_{18}

5 μm 4.6 × 250 mm column with dinethylolctadecylsilyl bonded amorphous silica and Waters 2487 dual λ absorbance detector was used. The mobile phase was water:methanol 70:30 (v:v) (HPLC Grade; J.T. Baker) with 0.1% formic acid (98%; J.T. Baker) of both phases. A flow rate of 0.4 [mL/min] was used, and the absorbance was measured at 266 [nm].

4. References

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