



“your chromatography specialists”

BioTrap 500 MS

An HPLC online extraction column designed for MS

INSTRUCTION MANUAL

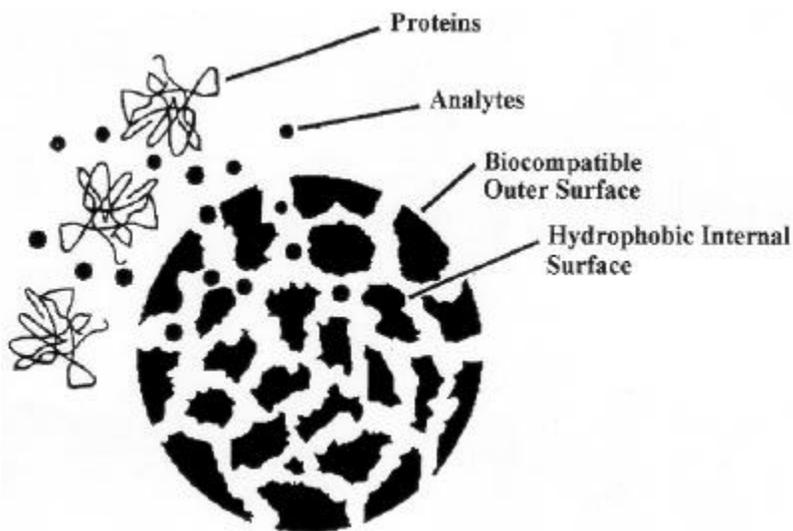


Illustration of a BioTrap 500 particle

Instruction manual BioTrap 500 MS

1

CONTENTS	Page
What is BioTrap 500 MS	1
Set-up of a HPLC column switching system.....	2
Connection of the columns to the 6-port switching valve	2
Description of the column switching system	2
How to use BioTrap 500 MS	3
Installation of the BioTrap 500 MS column.....	3
Sample preparation	4
Extraction mobile phase composition	4
General extraction methods	5
Analytical column and mobile phases	5
Injection volume, flow rates, extraction and elution times	6
Extraction	6
Elution of the extraction column.....	6
Reequilibration of the extraction column	6
Method development	7
Maintenance and column lifetime	7
Storage	7
Appendix	8
10-port switching valve.....	9
References	9
Applications	10
Product list.....	11

WHAT IS BIOTRAP 500 MS?

BioTrap 500 MS is a new biocompatible sample extraction column, enabling repeated direct injections of plasma, serum, milk, supernatants of cell cultures, fermentation broth and other complex matrices, into the HPLC-system without previous clean-up procedures (except for a simple centrifugation).

The BioTrap 500 MS is pH stable extraction column (pH 2-11, preferably 2-10 due to limitations in pH stability of certain parts in the HPLC equipment) with a biocompatible external surface and a hydrophobic internal surface (hydrophobic polymer). The biocompatibility has been obtained by attachment of the plasma protein α_1 - acid glycoprotein (AGP) on the external surface of the particles. AGP is an extremely stable protein which tolerates organic solvents used in reversed-phase HPLC. The surface within the pores is a hydrophobic polymer and the pores of the matrix are small enough to exclude the plasma proteins and other macromolecular compounds.

Detection methods as mass spectrometry, fluorescence, electrochemical detection and UV can be used. It is favourable to use a detection method giving as high detection selectivity as possible, such as MS or MS-MS.

By using a 6-port valve with an electric actuator and injecting the plasma sample with an autosampler, coupled to an extra pump for the extraction mobile phase, it is possible to obtain complete automation of the system.

Connection of the columns to the 6-port switching valve:

A 6-port valve is depicted in Fig. 1. The different components are connected to different ports in the valve as described below:

- Couple the extraction column between **port 3 and 6**
- Connect Pump A to the autosampler and the outlet of the autosampler to the filter. The filter is coupled to **port 1**.
- Connect a tubing from **port 2** to waste.
- Connect Pump B to **port 4**.
- Couple the inlet of the analytical column to **Port 5**.

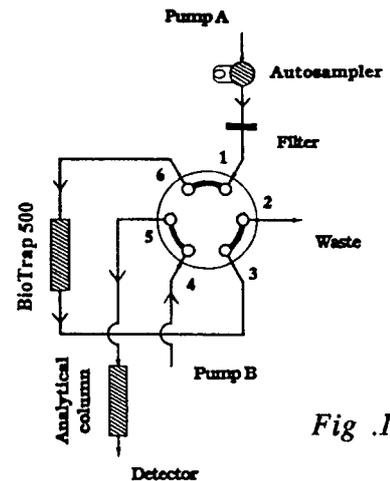


Fig .1

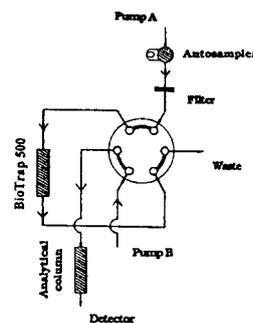
All tubing where plasma is transported, ie.from the autosampler to the extraction column to the waste tubing, should be made of tubing with an ID of at least 0.5 mm.

Description of the column-switching system

Pump A is pumping the extraction mobile phase through the autosampler, where the sample is injected. After the autosampler a filterholder is inserted, with a 2 µm biocompatible filter. After the passage of the filter, the sample is transported to the extraction column via the 6-port valve (**extraction position**). **Pump B** is pumping the analytical mobile phase through the analytical column via the 6-port valve (**extraction position**).

In **the elution position** the flow from pump A (the extraction mobile phase) is going to waste. The flow from pump B (the analytical mobile phase) is backflushing the extraction column into the analytical column.

Extraction position



Elution position

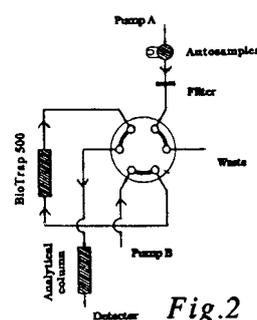


Fig.2

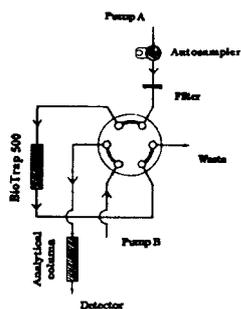


Fig.3

1. Extraction position - injection and separation of the analyte(s) from high molecular weight compounds (e.g. proteins).

In this position the sample (e.g. plasma) is injected onto BioTrap 500 MS (the extraction column). The plasma proteins and other macromolecular compounds will be excluded from the pores and eluted to waste. Low molecular weight compounds penetrate the pores of the particles and are retained by the hydrophobic inner surface of the particles. When the proteins have been washed out from the column, the valve can be switched to the elution position.

Elution position

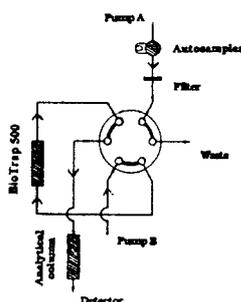


Fig.4

2. Elution position - transfer of the adsorbed compounds from the extraction column to the analytical column. In this position the analytes will be eluted from the extraction column by the analytical mobile phase. When the analytes have been transferred to the analytical column, the valve can be switched back to the extraction position.

Extraction position

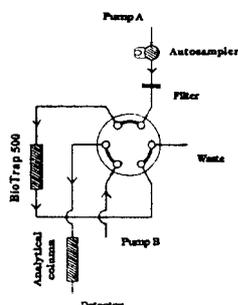


Fig.5

3. Extraction position - the final separation of the analyte(s) on the analytical column and reequilibration of the extraction column. In this position the final separation of the analytes is achieved on the analytical column. Simultaneously, the extraction column is reequilibrated with the extraction mobile phase and the system will be ready for the next injection.

INSTALLATION OF THE BIOTRAP 500 MS COLUMN

Equilibrate the column with the analytical mobile phase at a flow rate of 1 ml/min for 45 minutes. Equilibrate the column with the extraction mobile phase for about 2 minutes (3.2 ml/min). When changing the analytical mobile phase it is advised to wash the extraction column for about 30 minutes (1 ml/min) with this mobile phase.

It is recommended to use our specially designed 2 µm filter (art.no. F-117), with filter holder in PEEK (art.no. F-114), coupled on-line in front of the BioTrap 500 MS column (see Fig. 1), in order to protect the column from particulate impurities.

At least one plasma injection should be made prior to regular use.

Please note that there is a notch in the column under the label, where a wrench can be inserted.

The samples should be centrifuged before injection to remove cryoproteins and other particulate impurities. After centrifugation the cryoproteins will be present as a white or light yellow layer on top of the plasma. Other particulate impurities may be present in the bottom of the tube. **Note!** Be sure that the washing liquid in the autosampler does not precipitate the plasma proteins. As for example 5% 2-propanol in distilled water can be used.

The use of a guard column is also recommended in order to protect the analytical column.

EXTRACTION MOBILE PHASE COMPOSITION

The general rule is to choose a pH of the extraction mobile phase which gives the analyte as low charge as possible in order to obtain high recovery. **Be sure that the extraction mobile phase is compatible with plasma/serum or other biological samples.** Avoid pH 3-5 for extraction since plasma proteins can be precipitated in this pH range. To test the biocompatibility of the extraction mobile phase (if other extraction mobile phases than the general methods described below are used), mix 0.5 ml of centrifuged plasma with 0.5 ml of the extraction mobile phase in a tube. For comparison, make also another sample with 0.5 ml plasmalserum mixed with 0.5 ml distilled water. After 15 minutes the plasma solution should be as clear as before adding the buffer.

Buffer concentrations between 10-100 mM can be used, however 10-50 mM are mostly used. An organic solvent should be added to the extraction mobile phase in order to improve the washing of the extraction column and to displace the drug from the plasma protein binding. The solvent can be 2-propanol, acetonitrile or methanol. The recovery is affected by the organic solvent concentration.

Below is the maximum concentrations of three organic solvents that can be used in the extraction mobile phase.

<u>Solvent</u>	<u>Max. concentration in the extraction mobile phase</u>	
2-propanol	5%	(used at pH 2 - 2.6 and pH 6 - 10)
Acetonitrile	10%	(used at pH 6 - 10)
Methanol	10%	(used at pH 6-10)

Note that only 2-propanol can be used at low pH !!!!!

General extraction methods have been developed. These methods work for an extremely broad range of compounds. However, for some compounds it could be necessary to adjust the conditions, for example lower 2-propanol concentration.

1. Extraction of basic compounds

Extraction mobile phase:

4% 2-propanol in 10 mM ammonium acetate buffer pH 10.0 (pH adjusted with ammonium hydroxide).

2. Extraction of acidic compounds

4% 2-propanol in 100 mM formic acid.

ANALYTICAL COLUMN AND MOBILE PHASES

Basic drugs are extracted at pH 10 which means that when the sample is transferred from the extraction column to the analytical column, a plug with high pH will enter the analytical column. Basic drugs are chromatographed on the analytical column using a mobile phase with a pH of 2-3. **It is important that the buffer is strong enough to reduce the pH of the high pH plug transferred from the extraction column. Otherwise the lifetime of a silica based analytical column can be reduced.** When MS detection is used the analytical mobile phase is normally **50 mM formic acid** mixed with an organic solvent. This is enough to reduce the pH of the basic plug. **Another possibility is to use a pH stable silica column or a polymer based analytical column.**

INJECTION VOLUME, FLOW RATES. EXTRACTION AND ELUTION TIMES

Up to 500 μ l of plasma can be repetitively injected. The recovery might be affected by the injection volume. **The 20x4.0 mm column is recommended** as the starting column. For smaller sample volumes and if the compound has a low degree of protein binding and/or high affinity to the extraction column the 13x4.0 mm column can be used. When using micro analytical columns the BioTrap micro extraction column (20 x 2.0 mm) can be used.

Extraction

The column can be used up to 5 ml/min. Recommended flow rate is 3.2 ml/min. The extraction time is dependent on the injection volume. Examples of extraction times are given below for plasma/serum injections using an **extraction flow of 3,2 ml/min.**

<i>Injection volume (μl)</i>	<i>Extraction time (min)</i>
10 - 25	0.5 - 1
50 - 100	1 - 1.5
100 - 250	1.5 - 2
250 - 500	2.5

See also applications below.

Elution of the extraction column (backflush)

A flow rate of 1 ml/min is recommended if a 4.6 mm i.d. analytical column is used. The elution time is dependent of injected volume and the eluting strength of the analytical mobile phase. Normally 3-4 minutes elution time is used. See also applications below.

Reequilibration of the extraction column

After elution of a sample and prior to injection of a new sample the extraction column must be equilibrated with the extraction mobile phase. An equilibration time of 1-2 min using a flow of 3.2 ml/min is recommended.

Note! Plasma samples must be centrifuged before injection. See also instructions under "Sample preparation".

- 1. Choose a detection method** that gives high detection selectivity (MS or MS-MS) and high enough sensitivity.
- 2. Develop a preliminary analytical method**, by choosing a column and a mobile phase composition giving good chromatographic performance. If the analyte is a basic compound, we recommend the use of 50 mM formic acid with appropriate concentration of organic modifier.
- 3. Connect the analytical column and the extraction column to the six-port switching valve** according to Figs. 1 and 2. Use the extraction mobile phases recommended above under "General extraction methods".
- 4. Optimization of the coupled column system.**

Optimize the analytical method to avoid interferences by changing the mobile phase composition, changing the analytical column to one with different surface chemistry or by changing the detection method.

In some cases the general extraction mobile phase might be slightly changed in order to obtain high enough recovery. This can be done by adjusting the organic modifier concentration, the buffer concentration or the pH. See above under "Extraction mobile phase composition".
- 5. Validate the method.**

MAINTENANCE AND COLUMN LIFETIME

The lifetime of the BioTrap 500 MS column is dependent of the sample matrix, the injected volume, the composition of the mobile phases and how the extraction column is rinsed by the extraction mobile phase and by the analytical mobile phase.

Under optimal conditions more than 100 ml of plasma can be injected on the same column. It is advisable to change the analytical guard column regularly. The filter in front of the extraction column should be exchanged when about 50 ml of plasma/serum has been injected.

STORAGE

The extraction column can be stored at room temperature. When not in use it is recommended to fill the column with 15% 2-propanol in distilled water.

APPENDIX

Above we have discussed the use of the BioTrap 500 MS column in combination with the mass spectrometer (MS) as the detector. However, the column can also be used in other types of systems where other detectors than the MS is used (detectors like fluorescence detectors, electrochemical detectors as well as UV). Using detectors of that type means that many different types of mobile phases can be used, such as non volatile phosphate buffers etc. For this type of applications see the applications on the website: <http://www.chromtech.se/biotrap> or the Application notes for BioTrap 500 C8 and C18 (can be ordered from Chromtech or your local distributor).

A short description of other techniques which can be used with the BioTrap 500 MS column, using other detectors than MS, is given below. See also the above website or the application notes. References to published papers are also given below.

1. The ionpair technique

A strategy has been developed to obtain high separation efficiency for basis analytes and to increase recovery for more hydrophilic basic analytes, when using the BioTrap 500 columns. These effects are created by the addition of an ionpairing agent, for example the sodium salt of an alkylsulfonic acid, in the mobile phases. Very low concentrations of the ionpairing agent is used, i.e. 1 - 10mM. Normally 5 mM of the ionpairing agent is added to the extraction mobile phase and 2 mM to the analytical mobile phase. By using higher concentration of the ionpairing compound in the extraction mobile phase, an enrichment effect on the top of the analytical column can be obtained. A volatile ionpairing agent, which can be used with MS-detection is pentafluoro propionic acid.

2. The displacement technique.

By adding a compound to the extraction mobile phase which displace the analyte from the plasma proteins binding, an increase in the recovery can be obtained.

An acid can be extracted in two different ways, as the uncharged acid and as an ionpair. Normally a very good recovery can be obtained by extraction of the uncharged acid at low pH (pH 2 - 2.6). Another way to increase the recovery is to use neutral pH and extract the acid as an ionpair with positively charged buffer ions like sodium, with a hydrophobic acid (for example octanoic acid) present in the extraction mobile phase. The hydrophobic acid will compete with the analyte for the protein binding and displace the analyte. This can also have a very positive effect on the efficiency. Furthermore, in this way the peaks can be compressed and the recovery can increase. This is very favourable in low concentration analysis. Such results are shown on the website: <http://www.chromtech.se/biotrap> or in the ChromTech Application Note no. 15 for ibuprofen.

Note!!! When using charged organic modifiers in the mobile phases the column should only be used with this type of modifier since charged modifiers may be very difficult to remove totally from the column. Therefore the properties of the column may be changed.

By using a 10-port valve, faster bioanalysis can be achieved. By connecting two BioTrap 500 columns in a 10-port valve (2-position), it is possible to handle two samples simultaneously. Fig. 6 shows the connection of two BioTrap 500 columns and one analytical column in one HPLC system.

In the first position, the sample is extracted on the BioTrap 500 column 1, while the previous sample is backflushed with the analytical mobile phase from BioTrap 500 column 2 onto the analytical column. In the second valve position, the sample extracted on BioTrap 500 column 1 is backflushed onto the analytical column, while the next sample is injected onto BioTrap 500 column 2 for extraction.

First position

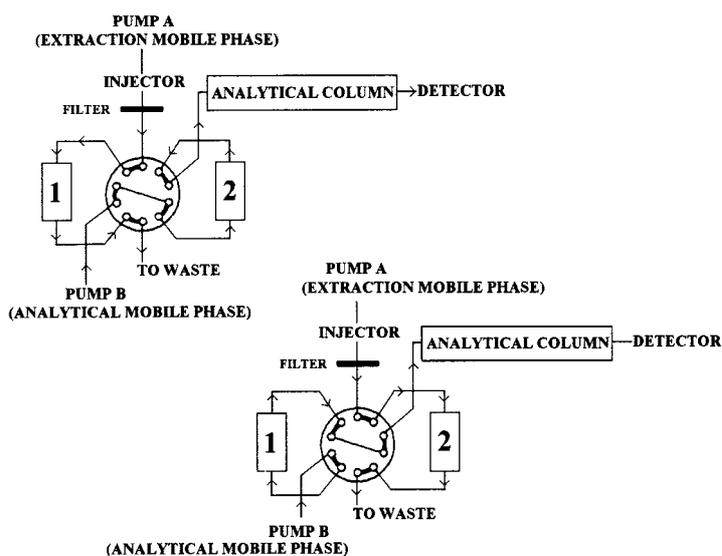
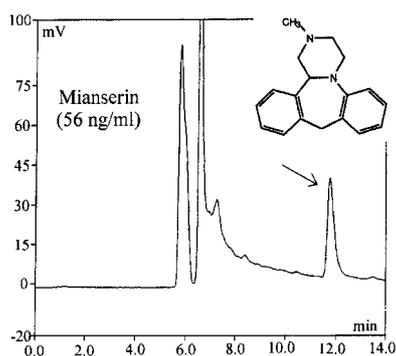


Fig. 6

References

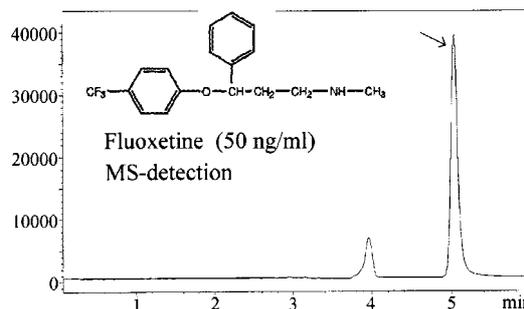
1. J. Hermansson, A. Grahn and I. Hermansson *J. Chromatogr.*, 660/1-2, 119 (1994)
2. J. Hermansson, A. Grahn and I. Hermansson, *Current separations*, **16**, No. 2, 55 (1997)
3. J. Hermansson et al. presented at HPLC 97, Birmingham
4. ChromTech Application Note no. 14
5. ChromTech Application Note no. 15
6. J. Hermansson, A. Grahn and I. Hermansson, *J. Chromatogr.A*, **797**, 251 (1998)



Sample: Mianserin 56 ng/ml
 Injection volume: 500µl serum
 Extraction column: BioTrap 500 MS, 20x4.0 mm
 Extraction conditions: 4% 2-propanol in 10 mM ammonium acetate buffer, pH 10 (adjusted with ammoniumhydroxide)
 Extraction flow: 3.2 ml/min
 Analytical column: Hypersil Elite C18, 150x4.6 mm, 5 µm + guard, 10x4.0 mm, 5 µm
 Analytical mobile phase: 40% acetonitrile in 50 mM formic acid
 Flow: 1 ml/min
 Online postcolumn derivatization: Beam Boost UV=254 nm, 20 m x 0.3 mm I.D.
 Detection: Fluorescence: ex=270nm, em=430nm

Analysis Program

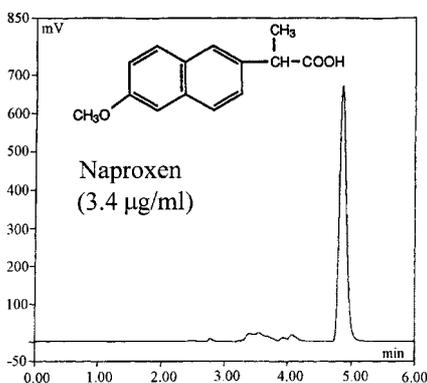
Extraction time 2.5 min
 Elution time 4 min
 Equilibration time 7.5 min



Sample: Fluoxetine 50 ng/ml
 Injection volume: 50µl serum
 Extraction column: BioTrap 500 MS, 20x4.0 mm
 Extraction conditions: 4% 2-propanol in 10 mM ammonium acetate buffer, pH 10 (adjusted with ammoniumhydroxide)
 Extraction flow: 3.2 ml/min
 Analytical column: Zorbax SB-CN, 150x4.6 mm, 5 µm + guard 12.5x4.6 mm, 5 µm
 Analytical mobile phase: 35% acetonitrile in 50 mM formic acid
 Flow: 1 ml/min
 HPLC system: HP 1100 Series
 Detection: HP 1100 Series LC/MSD, API-ES positive at 310.1

Analysis Program

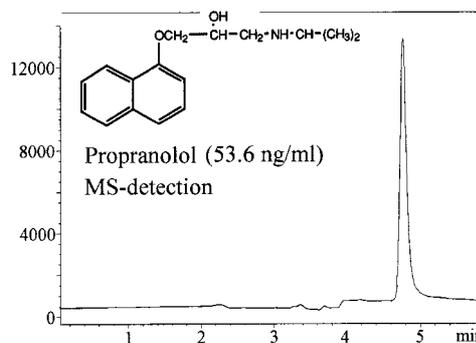
Extraction time 1 min
 Elution time 3 min
 Equilibration time 2 min



Sample: Naproxen 3.4 ng/ml
 Injection volume: 10µl serum
 Extraction column: BioTrap 500 MS, 20x4.0 mm
 Extraction conditions: 4% 2-propanol in 100 mM formic acid
 Extraction flow: 3.2 ml/min
 Analytical column: Zorbax XDB-C8, 150x4.6 mm, 5 µm + guard 12.5x4.6 mm, 5 µm
 Analytical mobile phase: 30% acetonitrile in 25 mM ammonium acetate
 Flow: 1 ml/min
 Detection: Fluorescence: ex=230 nm, em=350 nm

Analysis Program

Extraction time 1 min
 Elution time 3 min
 Equilibration time 2 min



Sample: Propranolol 53.6 ng/ml
 Injection volume: 50µl serum
 Extraction column: BioTrap 500 MS, 20x4.0 mm
 Extraction conditions: 4% 2-propanol in 10 mM ammonium acetate buffer, pH 10 (adjusted with ammoniumhydroxide)
 Extraction flow: 3.2 ml/min
 Analytical column: Zorbax SB-CN, 150x4.6 mm, 5 µm + guard 12.5x4.6 mm, 5 µm
 Analytical mobile phase: 25% acetonitrile in 50 mM formic acid
 Flow: 1 ml/min
 HPLC system: HP 1100 Series
 Detection: HP 1100 Series LC/MSD, APCI positive at 260.3

Analysis Program

Extraction time 1 min
 Elution time 3 min
 Equilibration time 2 min

BioTrap Ordering Information

Part No. Description

BioTrap 500 MS

BMS134C	BioTrap 500 MS, 13x4mm 2 cartridges
BMS134K	BioTrap 500 MS, 13x4mm w/holder
BMS202C	BioTrap 500 MS, 20x2mm 2 cartridges
BMS202K	BioTrap 500 MS, 20x2mm w/holder
BMS204C	BioTrap 500 MS, 20x4mm 2 cartridges
BMS204K	BioTrap 500 MS, 20x4mm w/holder

BioTrap 500 MS Kits include:

BT-KIT10	13x4.0mm + 20x4.0mm + holder
BT-KIT11	13x4.0mm + holder + filter holder+ 5 filters
BT-KIT12	20x4.0mm + holder + filter holder+ 5 filters
BT-KIT13	13x4.0mm + 20x4.0mm + holder + filterholder in PEEK + 5 PEEK-filter
BT-KIT14	20x4.0mm + BioTrap 500 C18 20x4.0 mm + holder + filterholder in PEEK + 5 PEEK-filter
BT-KIT15	20x4.0mm + BioTrap 500 C8 20x4.0mm + holder + filterholder in PEEK + 5 PEEK-filter
BT-KIT16	20x4.0mm + BioTrap 500 C18 20x4.0mm + BioTrap 500 C8, 20x4.0mm + holder + filterholder in PEEK + 5 PEEK-filter

BioTrap 500 C18

B18134C	BioTrap 500C18, 13x4mm 2 cartridges
B18134K	BioTrap 500C18, 13x4mm w/holder
B18202C	BioTrap 500C18, 20x2mm 2 cartridges
B18202K	BioTrap 500C18, 20x2mm w/holder
B18204C	BioTrap 500C18, 20x4mm 2 cartridges
B18204K	BioTrap 500C18, 20x4mm w/holder

BioTrap 500 C18 Kits include:

BT-KIT1	13x4.0mm + 20x4.0mm + holder
BT-KIT2	13x4.0mm + holder + filterholder in PEEK + 5 PEEK-filter
BT-KIT3	20x4.0mm + holder + filterholder in PEEK + 5 PEEK-filter
BT-KIT4	13x4.0mm + 20x4.0mm + holder + filterholder in PEEK + 5 PEEK filter
BT-KIT9	20x4.0mm + BioTrap 500 C8 20x4.0mm + holder + filterholder in PEEK + 5 PEEK-filter

BioTrap 500 C8

B8134C	BioTrap 500C8, 13x4mm 2 cartridges
B8134K	BioTrap 500C8, 13x4mm w/holder
B8202C	BioTrap 500C8, 20x2mm 2 cartridges
B8202K	BioTrap 500C8, 20x2mm w/holder
B8204C	BioTrap 500C8, 20x4mm 2 cartridges
B8204K	BioTrap 500C8, 20x4mm w/holder

CH2	Holder w/endfittings 2mm, pk/2
CH4	Holder w/endfittings 4mm, pk/2
BTF-114	Filterholder in PEEK for BTF-117
BTF-117	PEEK-filter 2um 5 pcs.

BioTrap 500 C8 Kits include:

BT-KIT5	13x4.0mm + 20x4.0mm + holder
BT-KIT6	13x4.0mm + holder + filterholder in PEEK + 5 PEEK-filter
BT-KIT7	20x4.0mm + holder + filterholder in PEEK + 5 PEEK-filter
BT-KIT8	13x4.0mm + 20x4.0mm + holder + filterholder in PEEK + 5 PEEK filter

Electrically Actuated Valves

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EC10W	10 Port Electrically Actuated Valve

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