R-BIOPHARM RHÔNE LTD

KOBRA® CELL (Art. No.: K01)

Instruction manual

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1. Introduction to KOBRA® CELL

1.1 Intended use

Confirmation of the presence of aflatoxins in a sample by HPLC requires derivatisation of aflatoxins B1 and G1 in order to enhance their natural fluorescence and make them more easily detected. Previously, the only options available for derivatising aflatoxins involved the use of trifluoroacetic acid (TFA), pyridinium bromide perbromide (PBPB) or iodine. All of these methods have significant limitations which can be overcome by use of the KOBRA® CELL.

Pre-column derivatisation with TFA requires the solution containing aflatoxin to be blown to dryness under a stream of nitrogen, potentially leading to a loss of toxin. Further limitations are that the reaction takes 30 minutes at 50 °C, and the TFA reagent is itself corrosive and harmful to handle.

The post-column PBPB method involves addition of the diluted reagent into the eluate from the HPLC column. The limitations of this method are the use of a second pump and the difficulty in dissolving the PBPB as well as the hazardous nature of the reagent.

Post-column derivatisation with iodine also has some limitations including the need for a second pump, water bath or oven which can be expensive. It is necessary to clean the equipment regularly in order to avoid iodine crystals forming inside the reaction coil. Finally, the iodine must be prepared fresh each day due to its unstable nature.

The KOBRA® CELL overcomes the problems relating to alternative derivatisation procedures. It is an electrochemical cell connected to an HPLC system downstream from the HPLC column and in line with the column effluent and the fluorescence detector. The KOBRA® CELL generates a reactive form of bromine for derivatisation of aflatoxins B1 and G1, resulting in enhanced fluorescence and thus more sensitive detection.

The KOBRA® CELL is used by hundreds of labs around the world and is mentioned in several EU and other international standard methods.

1.2 Derivatisation reaction

The aflatoxins and the mobile phase enter the KOBRA® CELL and the electrochemical reaction occurs generating the reactive form of bromine. The reaction between the reactive bromine and the aflatoxins must take place before the derivatised aflatoxins enter the fluorescence detector. Hence the length of the reaction coil is critical. A minimum reaction time of 4 seconds is required.

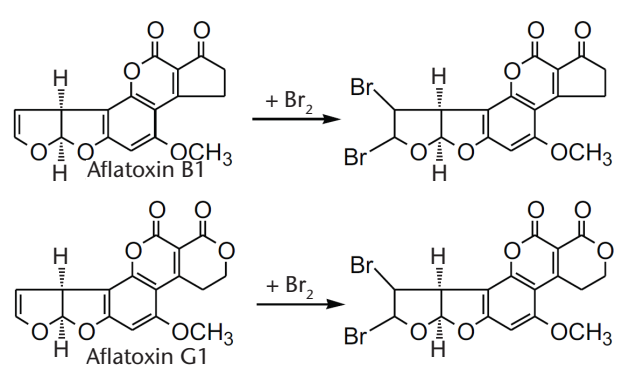


Fig 1.: Derivatisation reaction

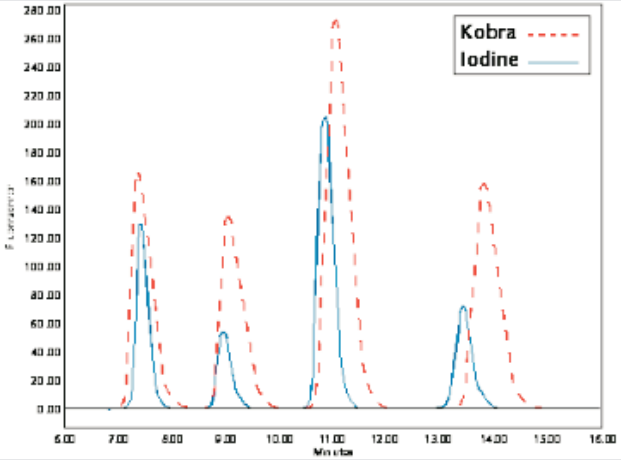


Fig 2.: Comparison of derivatisation methods

1.3 Amplification of aflatoxins

The KOBRA® CELL can be expected to amplify the signal of aflatoxin G1 and B1 by approximately 37 and 21 times respectively.

2. KOBRA® CELL contents

2.1 Contents

- 1 x KOBRA® CELL
- 1 x Power Pack (including 1 red and 1 black connection lead)
- 1 x Electrical adapter (with various adapters)
- 1 x 1 m length of 0.5 mm ID PEEK™ tubing
- 1 x Spare membrane

2.2 Reagents required

- Distilled Water
- Methanol
- Potassium Bromide
- Nitric Acid

2.3 Accessory products

- 1 x KOBRA® CELL Installation Pack (Product Code: K03)*
- Guard column: Inertsil ODS-3, 5 µm, 4 mm x 10 mm or equivalent

* Available from R-Biopharm. Please contact your local distributor for further information.

2.4 Hazards

Mycotoxins are very hazardous substances. Only laboratories equipped to handle toxic materials and solvents should perform analyses. Suitable protective clothing, including gloves, safety glasses and lab coats should be worn throughout the analysis.

2.5 Decontamination

Prior to disposal, excess standard solutions should be treated with at least one-tenth their volume of 5 % sodium hypochlorite. Labware and contaminated waste should be immersed in 5 % sodium hypochlorite solution for 30 minutes followed by the addition of 5 % acetone for 30 minutes. Flush with copious amounts of water before disposal. After decontamination labware should be thoroughly washed. Incinerate waste if regulations permit.

3. Assembly of the KOBRA® CELL

3.1 Components

	Art. No.	Description
1	N/A	6 locating screws
2	N/A	White plastic housing with red terminal (top)
3	N/A	Storage plugs & capillary tubing (for when KOBRA® CELL is not in use)
4	K09	PTFE spacer: 0.1 mm with contact area
5	K05	Platinum electrode
6	K07	PTFE spacer: 0.25 mm with reaction area
7	K02	Ion exchange membrane
8	K07	PTFE spacer: 0.25 mm
9	K10	Spacer grid
10	K07	PTFE spacer: 0.25 mm with reaction area
11	K04	Stainless steel electrode
12	K08	PTFE spacer: 0.1 mm with contact area
13	N/A	White plastic housing with black terminal (bottom)

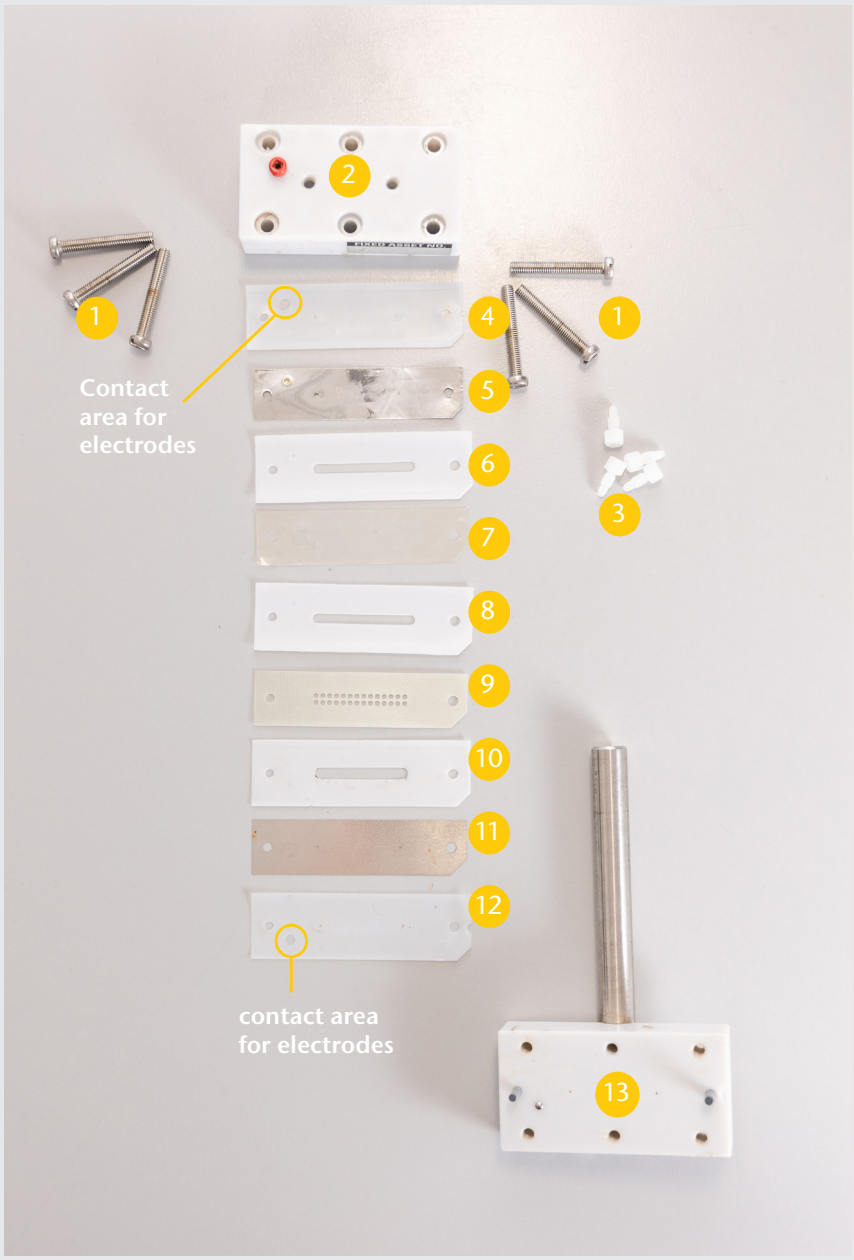


Fig 3.: KOBRA® CELL components

3.2 Precautions when assembling

- To reassemble the KOBRA® CELL it is important to tighten the locating screws at opposite corners before tightening the middle screws. This will ensure that the KOBRA® CELL is correctly assembled and will not leak.
- Do not over tighten locating screws as this will damage the threads.
- Never use metal nuts or ferrules directly with the KOBRA® CELL.
- Use a guillotine tubing cutter to prevent distortion of the diameter of the PEEK™ tubing used when assembling the KOBRA® CELL.

3.3 Assembly

Topside view of all KOBRA® CELL electrodes, membrane and spacers.

Smallest fitting hole for location peg on left side.

Largest fitting hole for location peg on right side.

Corner “cut out” on bottom right.

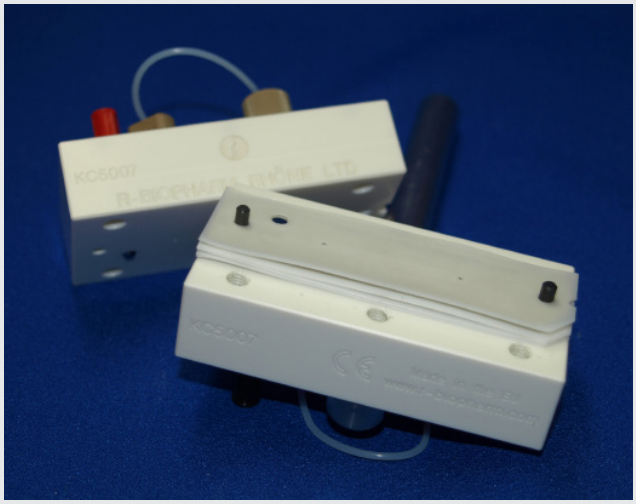


Fig 4.: Assembly of KOBRA® CELL

4. KOBRA® CELL installation

4.1 Power pack notes

The power pack is 9 V and is supplied with an adapter for use in the UK, USA, Europe and Australia. The plug can be converted by depressing the button located at the top of the adapter using a screwdriver. This releases the adapter that is in position. Slide to remove. To attach the required adapter place on the two pins and slide to connect. The power pack can now only be used at 100 µA and there is a light to show it has been switched on (top right). There is also an error light to indicate if there is a problem (bottom left).

4.2 Power pack technical specification

Power supply	Primary 110 - 240 V Secondary 9 V
Exit power current source	100 µA
Maximum Output Power	9 V
Loading time NICAD 9 V	Approximately 1 hour 15 minutes
Accuracy	0.5 % (This is an improvement on the previous model which was 2 %)
Working time with battery	20 hours
Approval	Current source has been manufactured to comply with European Directives and transmuted UK Legislation 73/23/EEC and 89/336/EEC. These directives relate to low voltage and electromagnetic compatibility, respectively. Two-pin mains connecter has been manufactured to comply with CE and UKCA Mark directives.

4.3 Installation guide

Prior to installation it is recommended to check the pressure of the KOBRA® CELL and the tubing intended to be used by installing the KOBRA® CELL directly to the HPLC pump, bypassing the analytical column and fluorescence detector. The mobile phase and flow rate intended to be used should be applied during this check.

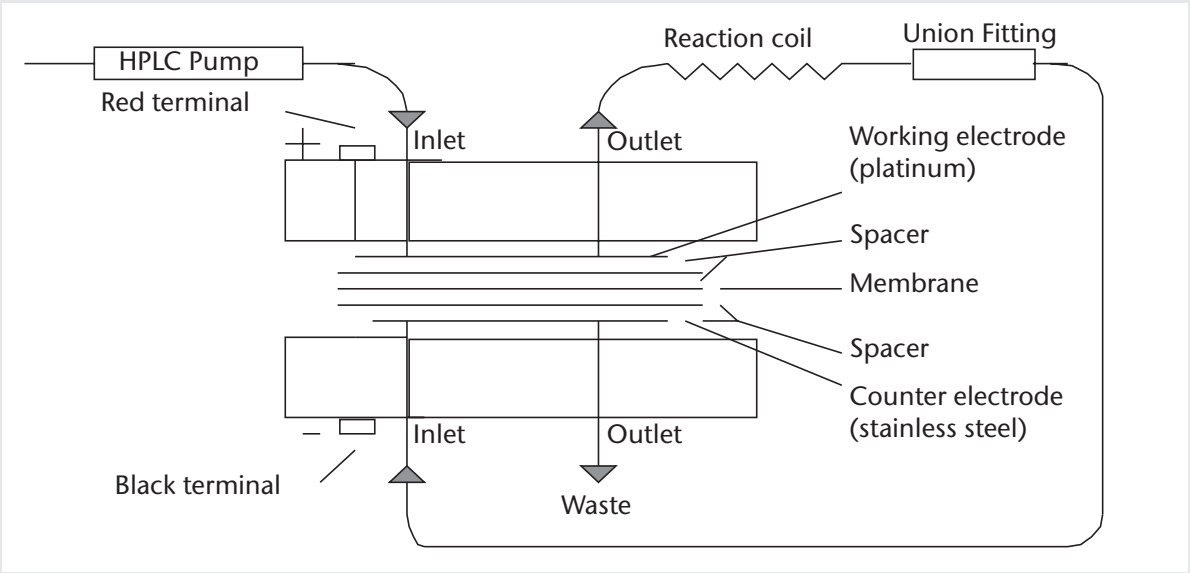
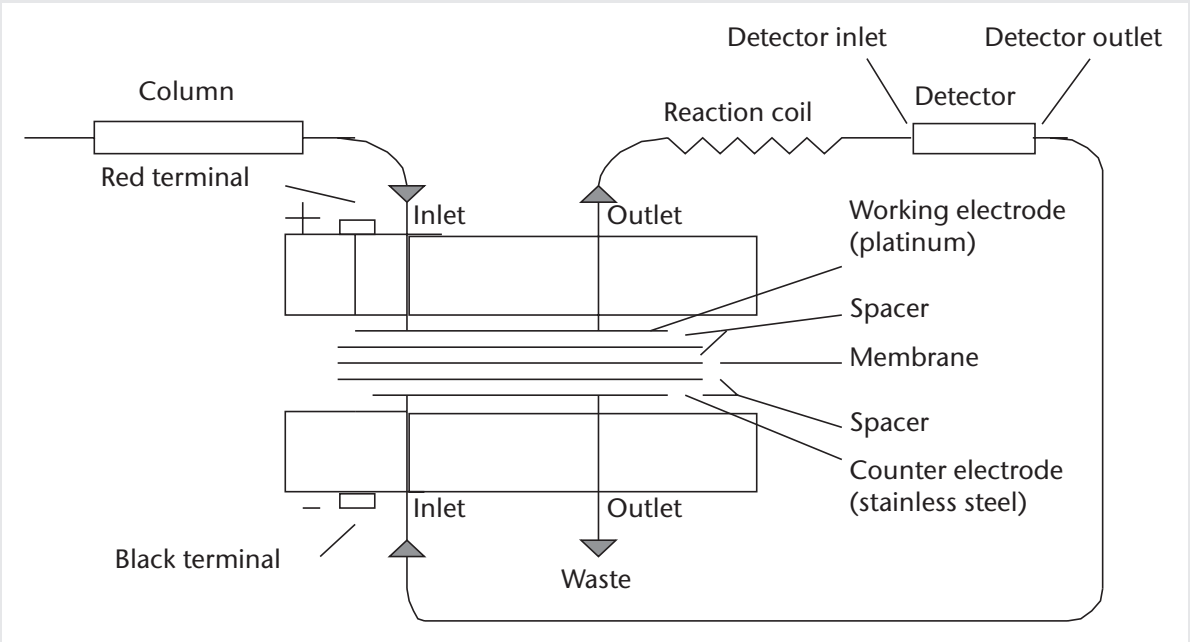


Fig 5.: KOBRA® CELL set up for pressure check



Membrane:	Reichelt Thomapor Anion.
Capillary connections:	Valco 1/16 inch (do not use metal nuts or ferrules because these can damage the electrochemical properties of the cell).
Working pressure:	Max 10 bar.

Fig 6.: KOBRA® CELL installation

4.4 Installation instructions

Caution:

- Do not over tighten.
- Cell connections should be made with plastic ferrules, not metal.
- KOBRA® CELL may only be used in horizontal position.

1. Unpack the KOBRA® CELL and check that all of the components are present.

2. Add 119 mg of potassium bromide and 350 µl of 4 M nitric acid to 1 litre of mobile phase.

3. Immerse the tubing from the HPLC pump into the mobile phase.

4. Disconnect the storage plugs and capillary tubing from the KOBRA® CELL. Connect the HPLC column discharge to the KOBRA® CELL inlet using plastic ferrules (hand tightened) and a length of PEEK™ tubing supplied with the kit.

5. Connect the KOBRA® CELL outlet using ferrules (hand tightened) and a length of PEEK™ tubing to the detector inlet according to the table below.

Caution:

- Use a guillotine tubing cutter to prevent distortion of the diameter of the tubing.

6. Connect the detector outlet to the KOBRA® CELL inlet using plastic ferrules (hand tightened) and PEEK™ tubing.

7. Connect the KOBRA® CELL outlet to waste using plastic ferrules (hand tightened) and PEEK™ tubing.

8. Switch on the HPLC pump and flush mobile phase through the system for approximately 5 minutes.

9. Connect the KOBRA® CELL power pack to the cell, red lead to red terminal and black lead to black terminal.

Caution:

- Do not switch on without mobile phase flowing through the KOBRA® CELL or the membrane will be damaged.

10. After the HPLC has been allowed to stabilise (approximately 30 minutes) the KOBRA® CELL is ready to use.

Determination of reaction coil / PEEK™ tubing length							
Flow Rate		0.5 ml / min	0.6 ml / min	0.7 ml / min	0.8 ml / min	0.9 ml / min	1.0 ml / min
Internal diameter of reaction coil / PEEK™ tubing (ID)	0.2 mm	106.1 cm	127.3 cm	148.5 cm	169.8 cm	191.0 cm	212.2 cm
	0.25 mm	67.9 cm	81.5 cm	95.1 cm	108.6 cm	122.2 cm	135.8 cm
	0.4 mm	26.5 cm	31.8 cm	37.1 cm	42.4 cm	47.7 cm	53.1 cm
	0.5 mm	17.0 cm	20.4 cm	23.8 cm	27.2 cm	30.6 cm	**34.0 cm**
	0.6 mm	11.8 cm	14.1 cm	16.5 cm	18.9 cm	21.2 cm	23.6 cm
	0.8 mm	6.6 cm	8.0 cm	9.3 cm	10.6 cm	11.9 cm	13.3 cm
	1.0 mm	4.2 cm	5.1 cm	5.9 cm	6.8 cm	7.6 cm	8.5 cm

4.5 Precautions to take before running the KOBRA® CELL

- This device has only to be used with the supplied power pack.
- Always ensure there is a mobile phase flowing through the KOBRA® CELL before switching on the current source.
- Do not switch on without mobile phase flowing through the KOBRA® CELL or the membrane will be damaged.
- It is advised to run the mobile phase through the KOBRA® CELL for 30 minutes prior to analysis.
- Always turn off the KOBRA® CELL power pack before turning off the HPLC pump.
- Always use HPLC grade solvents (with minimal benzene content) from a quality supplier and maintain fresh supplies of potassium bromide.
- Never flush 100 % solvent through the KOBRA® CELL as this can damage the membrane.

4.6 Operating the KOBRA® CELL

The KOBRA® CELL power pack is turned on by moving the switch to the ‘On’ position. To shut down the device move the switch to the ‘Off’ position.

4.7 Error LED

- The LED will light up if there is no KOBRA® CELL attached to the current source.
- The LED will also light up when the KOBRA® CELL is dry and the flow of the current source is inhibited.

Fig 7.: Power pack

4.8 CHRONECT Symbiosis RIDA®CREST Specific Considerations

- Install a KOBRA® CELL onto the CHRONECT Symbiosis RIDACREST® system using blue PEEK™ tubing (0.25mm ID) from the HPLC column and from the KOBRA® CELL to the detector (reaction coil). From the detector outlet to KOBRA® CELL use green PEEK™ tubing (0.76mm ID) and KOBRA® CELL to waste use clear PTFE tubing to reduce the backpressure applied to the flow cell in the fluorescence detector.
- When using blue PEEK™ tubing (0.25mm ID) for the reaction coil, cut to the appropriate length considering the flow rate:
  - 1.1 ml/min = 149.4 cm
  - 1.2 ml/min = 163 cm
- It is recommended to replace the membrane if peak broadening is observed. This is an indicator that the membrane may be close to failure.
- Before switching to IMMUNOPREP® ONLINE OCHRATOXIN HPLC conditions, prime the KOBRA® CELL with 50 % methanol prior to removal from the system. The KOBRA® CELL cannot be subjected to the mobile phase recommended for use with IMMUNOPREP® ONLINE OCHRATOXIN.
- The power pack supplied with the KOBRA® CELL does not turn off automatically with the completion of a sequence. Enable a “low flow” (0.1 ml/minute) instrument method at the end of a sequence using the KOBRA® CELL to ensure the KOBRA® CELL does not dry out while the power pack is on.

5. HPLC conditions

HPLC conditions	
Derivatisation	KOBRA® CELL at 100 µA setting
Guard cartridge	Inertsil ODS-3 5 µm, 4 mm x 10 mm or (Hichrom) equivalent
Analytical column	Inertsil ODS-3V 5 µm, 4.6 mm x 150 mm (Hichrom) or equivalent
Mobile phase	Water : Methanol (60 : 40 v/v) Add 119 mg of potassium bromide and 350 µl 4 M Nitric Acid to 1 litre of mobile phase
HPLC pump	To deliver mobile phase
Flow rate	1.0 ml/minute
Fluorescence detector	Excitation: 362 nm
	Emission: 425 nm (B1 and B2) 455 nm (G1 and G2)
Column heater	Maintain guard and analytical columns at 40 °C
Integrator / data control system	From preferred supplier
Injector	Autosampler / Reodyne valve
Injection volume	100 µl
Elution order	G2, G1, B2, B1

The KOBRA® CELL is also suitable for use under the BS EN 14123:2007 conditions.

Avoid use of acetonitrile in mobile phase. The membrane has a low tolerance to acetonitrile, and if over-exposed will significantly reduce the effective lifespan. Continuous use of acetonitrile may also induce sudden failure of membrane.

6. KOBRA® CELL maintenance

6.1 Storage

- The KOBRA® CELL should be switched off and disconnected for washing and storage (Fig. 8).
- Always store the KOBRA® CELL filled with water in order to keep the membrane wet.

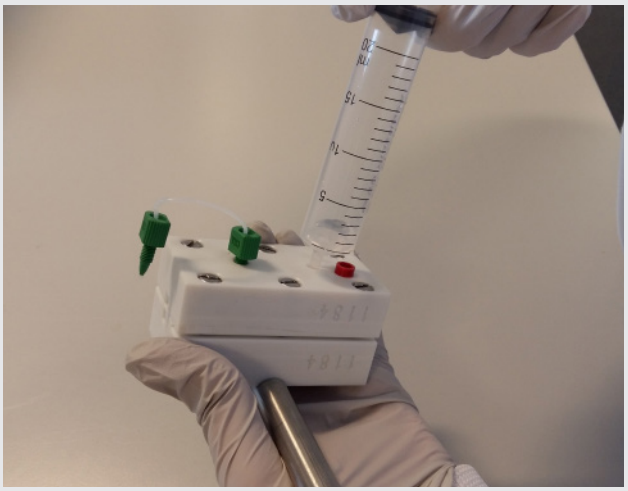


Fig 8.: Storage of KOBRA® CELL

To fill the KOBRA® CELL with water:

1. Fill a syringe with 5 - 10 ml of water and attach the tip of the syringe to a pipette tip.

2. Unscrew one of the storage plugs / ferrules from one inlet / outlet position of the KOBRA® CELL, leaving the other inlet / outlet positions sealed with storage plugs or ferrules.

3. Using the syringe with the pipette tip, insert this into the empty inlet / outlet position on the KOBRA® CELL and slowly fill the cell with water until water overflows from the syringe connection.

4. When finished, reinsert the storage plug / ferrule to contain the water within the cell and keep the membrane wet.

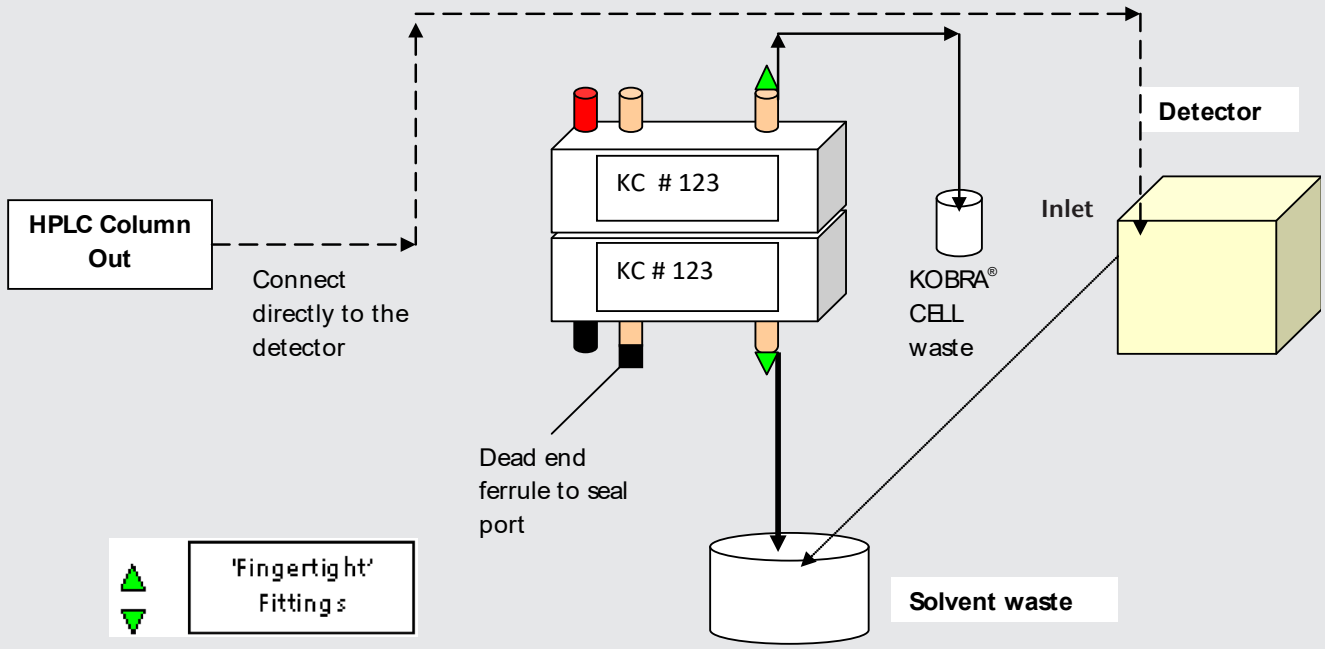


Fig 9.: KOBRA® CELL switched off for washing and storage

6.2 Daily cleaning

The HPLC system can be left running overnight at a reduced flow rate (e.g. 0.1 ml/min) and with the fluorescence detector and KOBRA® CELL power source turned off, if necessary. Alternatively, a better practice to prolong the life of the system is to clean each day, as follows:

1. Turn off KOBRA® CELL power pack.

2. Change the mobile phase to 50 % methanol 50 % water.

3. Switch on the HPLC pump and flush the system for at least 30 minutes before switching off the system.

4. The next day, change the mobile phase from 50 % solvent back to the normal mobile phase of potassium bromide and nitric acid, and flush the HPLC system through for at least 30 minutes before re-connecting the KOBRA® CELL to the power pack.

Caution:

- 100 % acetonitrile will damage the KOBRA® CELL membrane.

6.3 Monitoring performance

It is necessary to regularly monitor the performance of the KOBRA® CELL in order to detect any deterioration in the membrane contained within. The performance should be checked at the time of installation and then weekly by comparing the peak areas of a known aflatoxin standard. The same should also be done in order to monitor the deterioration of the lamp in the detector.

The pressure of the KOBRA® CELL and tubing intended to be used should be checked regularly (refer to section 4.3 for setup instructions). Monitoring the pressure of the equipment over time allows intervention before a damaging blockage forms.

Deterioration of the membrane will occur over a period of time depending upon the frequency of use and the type of samples analysed (Fig. 10). When the performance becomes unacceptable the membrane should be replaced (Product Code: K02).

Normally, it is found that even under extreme workloads the membrane does not need to be replaced for at least 6 months or 1,000 injections.

Fig 10.: Deterioration of KOBRA® CELL membrane

6.4 Changing the Membrane

1. Change the membrane when a decrease in performance is observed which cannot be attributed to the detector lamp. Additional membranes are available separately (K02).

2. Using a screw driver take off the white plastic housing of the KOBRA® CELL by removing the 6 locating screws.

3. Carefully separate the top white plastic housing, and remove the internal layers in turn using tweezers. Make a note of the position and orientation of each layer as it is removed.

4. Continue removing the internal layers until the membrane is exposed. Before removal inspect the membrane for damage. During the life of the KOBRA® CELL the bromination reaction causes the centre of the membrane to turn yellow in colour. No holes should be visible in the membrane other than the pre-cut holes for the locating pins at either end. Note the orientation of the membrane.

5. Remove the membrane from the KOBRA® CELL ready for replacement using tweezers.

6. Carefully remove the spare membrane, holding it by one end with the tweezers rather than the middle.

7. Position the replacement membrane over the different sized locating pegs, according to the size of pre-cut holes in the membrane.

Caution:

- Do not allow the membrane to dry out. Add distilled water if necessary.

8. Carefully replace the layers to be positioned on top of the membrane, remembering their correct order and orientation.

9. Secure the layers using the six locating screws.

7. Troubleshooting

7.1 No peaks observed

- If no peaks are observed on the HPLC chromatogram it is probably not the KOBRA® CELL that is at fault (Fig. 11). There may be a problem with a component of the HPLC system or with the standards used.
- Check that the bulb in the lamp does not need replaced. It should be replaced every 1,000 - 2,000 hours depending on the detector.
- Check there is not a problem with the detector.
- Check the standard has been prepared correctly, or the standard has not deteriorated. Standards should be prepared fresh every day.
- Check the PEEK™ tubing leading to and from the KOBRA® CELL is not blocked or distorted. Replace if required.
- Check the internal components of the KOBRA® CELL have been inserted in the correct order, particularly if no peaks are observed following the replacement of one of the components (Fig. 3).

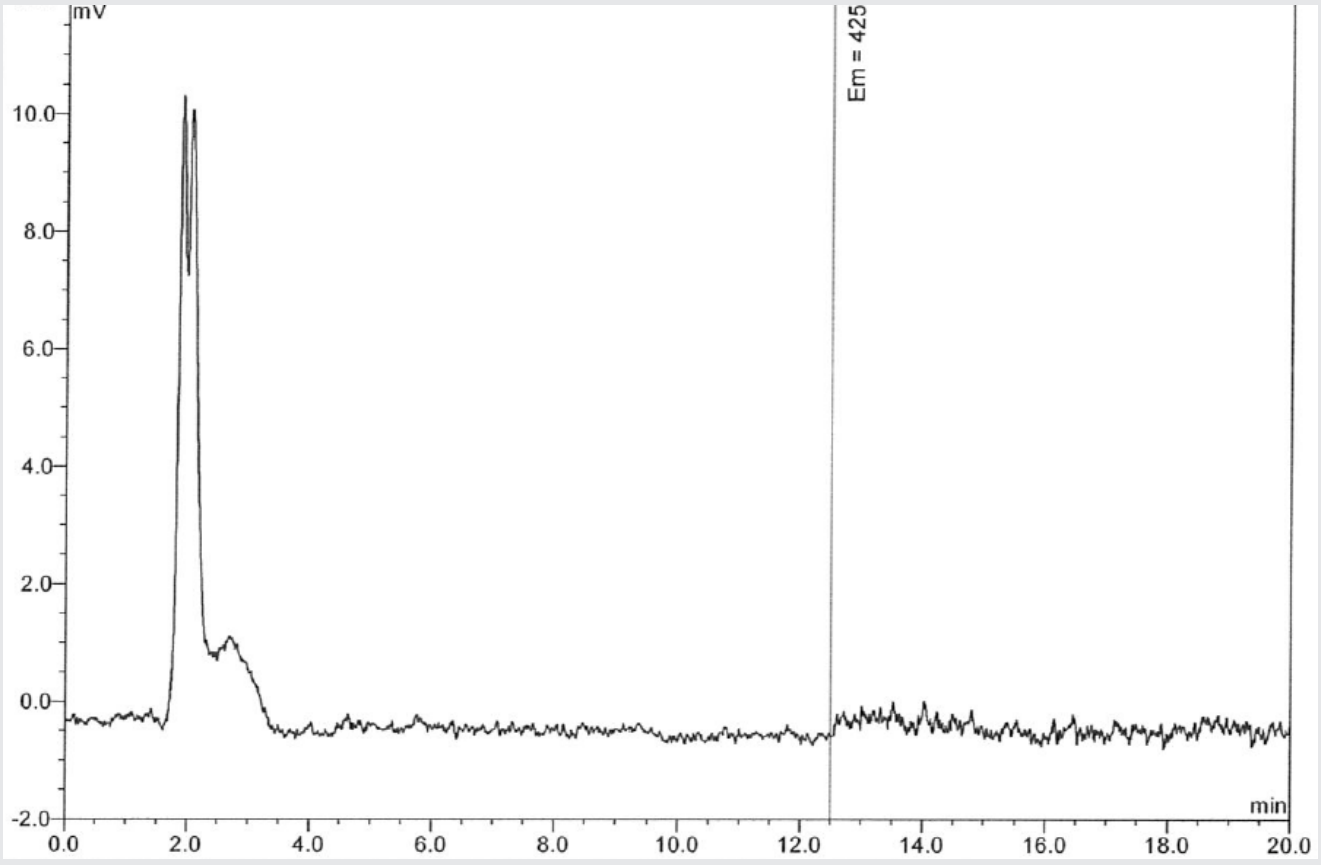


Fig 10.: No peaks obtained in chromatogram

7.2 Reduced / no aflatoxin B1 and G1 peaks observed

- Check the KOBRA® CELL is connected correctly to the HPLC (Fig 6).
- Check the connections to the KOBRA® CELL from the power pack.
- Check the KOBRA® CELL membrane. The membrane is the site of the actual derivatisation reaction (ion-exchange occurs on the membrane). The membrane starts a clear colour and changes to yellow in colour around the reaction centre. The colour change indicates that the membrane will need to be replaced.
- Check there is power in the power pack. Check
- Check there is power in the power pack. Check that the power pack has been turned on.
- Check the internal components of the KOBRA® CELL are in the correct order (Fig. 3), or that there is not a blockage in the system.
- No potassium bromide (or not enough potassium bromide) was added to the mobile phase. Prepare again.
- No nitric acid was added to the mobile phase. Prepare again.

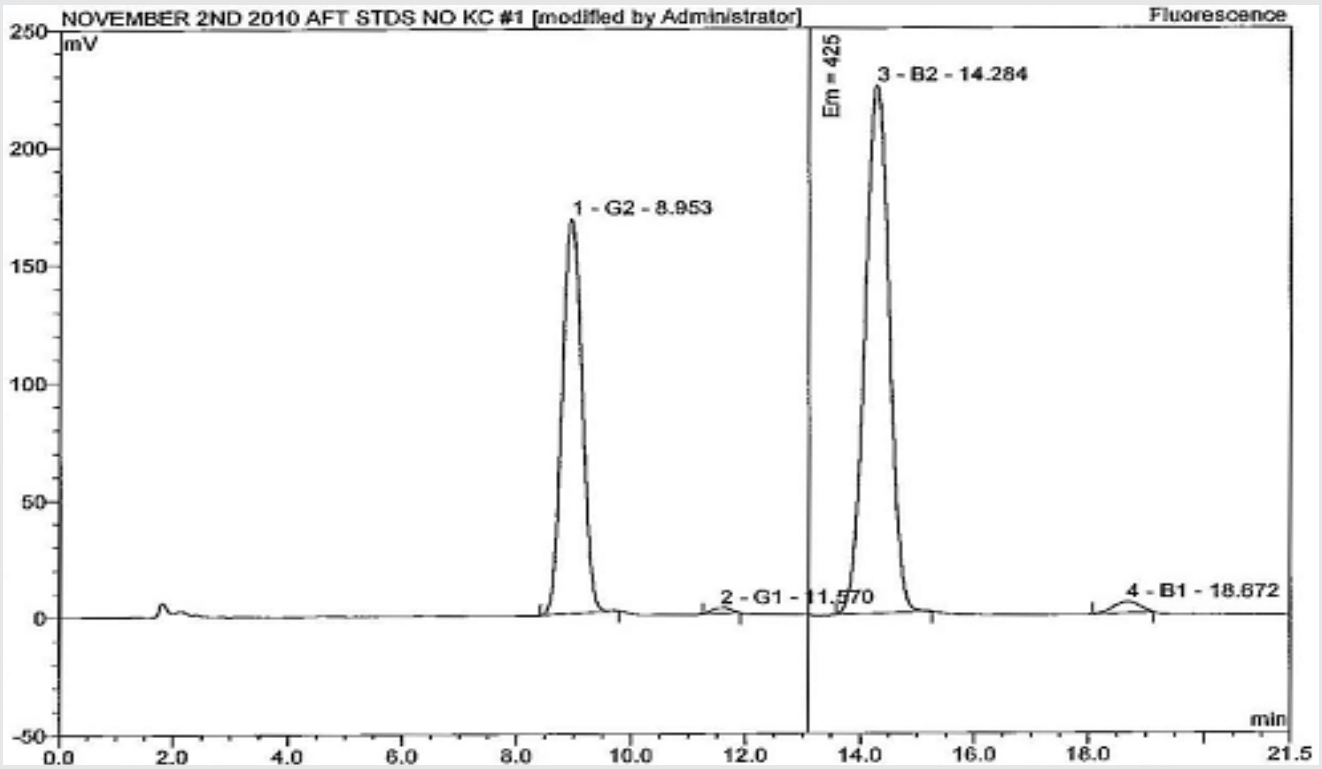


Fig 12.: Reduced / no aflatoxin B1 and G1 peaks observed

7.3 Abnormal Peaks Observed

- If abnormal peaks are observed on the HPLC chromatogram it is not normally the KOBRA® CELL that is at fault (Fig. 13).
- Check the correct mobile phase has been used.
- Check the correct HPLC column has been used.
- Check the internal components of the KOBRA® CELL have been inserted in the correct order (Fig. 3).
- Analytical column could need changed and may result in poor peak resolution or peak tailing.

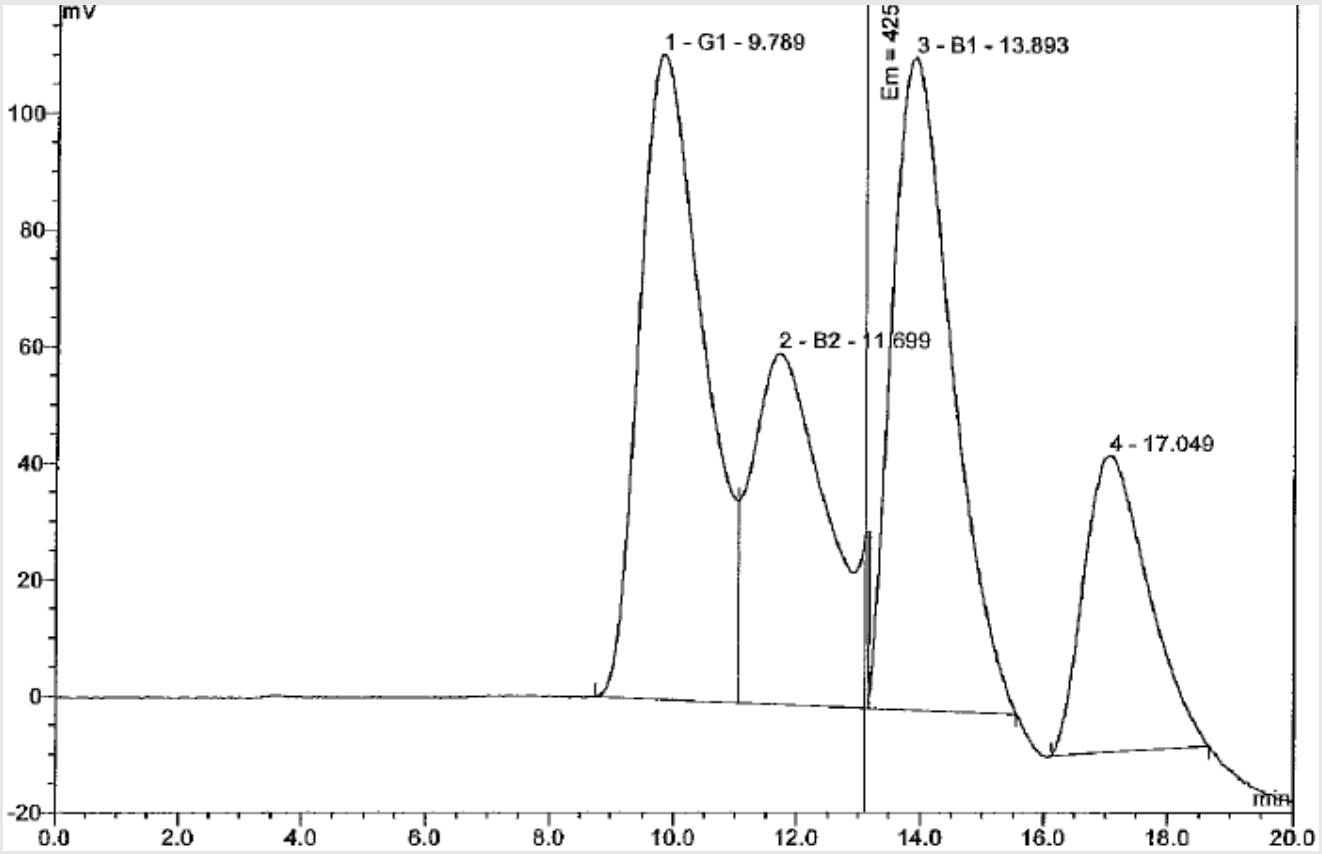


Fig 13.: Abnormal peaks obtained in chromato-

7.4 Error light observed on power pack

- The LED will light up if there is no KOBRA® CELL attached to the current source.
- The LED will also light up when the KOBRA® CELL is dry as the flow of the current source is inhibited.
- Check the red and black terminal leads are connected to the KOBRA® CELL correctly.
- Check the KOBRA® CELL has mobile phase running through it. It is important to keep liquid in the KOBRA® CELL at all times in order for it to function. It is advised to run mobile phase through the KOBRA® CELL for at least 30 minutes prior to injection of the standards or samples.
- Check the power pack. If a connection or component of the power pack is faulty the power pack should be replaced.

7.5 Leakage from KOBRA® CELL

- The locating screws on the KOBRA® CELL have not been tightened correctly. Please refer to 3.2, Precautions When Assembling.
- Check the locating screws on the KOBRA® CELL have not been over tightened causing damage to the threads. Once the threads are damaged a new white plastic housing is required.
- Check the PEEK™ tubing is not blocked or distorted. Replace if required.
- The PTFE spacers have distorted due to pressure, breaking the seal within the KOBRA® CELL. Disassemble the KOBRA® CELL and replace damaged parts.

7.6 KOBRA® CELL causing high pressure on the HPLC

- Check the PEEK™ tubing is not blocked or distorted. Replace if required.
- Check the inlet and outlet connections that the PEEK™ tubing is inserted into. If they are blocked they can be unscrewed and sonicated.
- Check the internal components in the KOBRA® CELL have been inserted in the correct orientation. Reassemble if required (Fig. 3 and 4).

7.7 Storage plug broken off or lodged in KOBRA® CELL

- Return to R-Biopharm Rhône for repair.

8. General Information

8.1 Quality

RBR products are developed, manufactured, tested and dispatched under an ISO 9001 registered Quality Management System, guaranteeing a consistent product, which always meets our performance specifications. Our products have been used in many collaborative studies to develop standard European and International Methods and are widely used by key institutions, food companies and government laboratories. Customer references for RBR products are available on request.

8.2 Technical support

RBR understand that from time to time users of our products may need assistance or advice. Therefore, we are pleased to offer the following services to our customers:

- Analysis of problem samples.
- Application notes for difficult samples.
- References from the RBR library.
- Installation and support of the KOBRA® CELL.
- Advice on detection parameters.
- Advice on preparation and handling of standards.
- Updates on legislation, sampling and other news by e-mail.
- Provision of spiked samples.

Please contact your local R-Biopharm distributor for further information.

8.3 Warranty

R-Biopharm Rhône Ltd makes no warranty of any kind, express or implied, except that all products made by R-Biopharm Rhône Ltd are made with materials of suitable quality. If any materials are defective, R-Biopharm Rhône Ltd will provide a replacement product. The user assumes all risk and liability resulting from the use of R-Biopharm Rhône Ltd products and procedures. R-Biopharm Rhône Ltd shall not be liable for any damages, including special or consequential damages, loss or expense arising directly or indirectly from the use of R-Biopharm Rhône Ltd products or procedures.