

## ACETYLCHOLINE AND CHOLINE

THE SMARTEST LC-EC APPLICATIONS FOR  
NEUROSCIENCE ANALYSIS  
EVER MASTERMINDED

## Monoamines and the metabolites

*Noradrenalin**Dopamine**Serotonin**5-hydroxyindole acetic acid (5-HIAA)**3,4-dihydroxyphenylacetic acid (DOPAC)**homovanillic acid (HVA)*

## OPA derivatized amines and amino acids

GABA and Glutamate

4-aminobutyrate (GABA)

Glutamate (Glu)

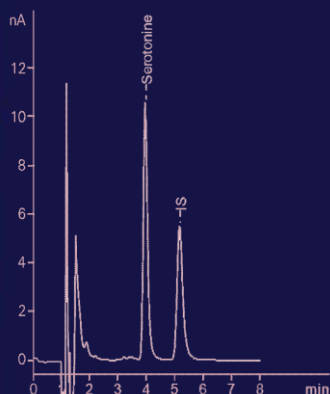
## Choline and Acetylcholine

*Choline (Ch)**Acetylcholine (ACh)*

## Markers for oxidative stress

*3-nitro-L-Tyrosine**8-OH-DPAT*

## Glutathione and other thiols



## INTRODUCTION

Determination of acetylcholine (ACh) by LC-EC is based on a reaction with acetylcholinesterase (AChE) and choline oxidase (ChO) [1]. Both enzymes are covalently bound to the stationary phase in a post column immobilised enzyme reactor (IMER). After separation, ACh is converted to choline by AChE and further oxidised by ChO. The resulting hydrogen peroxide can be detected electrochemically on a platinum working electrode or on a glassy carbon electrode coated with horseradish peroxidase (HRP). The ALEXYS<sup>®</sup> Acetylcholine analyzer featuring a FLEXCELL<sup>™</sup> with easily exchangeable working electrode disks can be used in combination with the ACh/Ch assay kit and peroxidase kit of BASi for sensitive detection of ACh and Ch.

- Extremely selective enzyme reactor
- Flexcell with easily exchangeable electrode
- Enzyme coating on WE for stable baseline
- Detection limit 10 fmol on column (2 nM/5 $\mu$ L)
- Total analysis time < 12 min

## Summary

## ALEXYS Ch and ACh Analyzer

A method is presented for analysis of Acetylcholine and Choline. Both are analysed by micro HPLC with an Immobilised Enzyme Reactor (IMER). The method is optimised for analysis of small sample volumes at nanomolar detection limits.



Fig. 1. ALEXYS Acetylcholine Analyzer

## Method

### LC-EC conditions

The enzymatic conversion of ACh and subsequent electrochemical detection of hydrogen peroxide is shown in Fig. 3. As the enzyme reaction takes place after HPLC separation, the method is suitable for determination of both Choline and ACh. Because the reaction with HRP is a reduction of peroxide, negative peaks will be measured therefore the detector polarity is reversed.

The IMER is connected directly to the FLEXCELL with a GC working electrode disc. The GC disk is coated with HRP according to the manual, corrected for the difference in diameter. Coating the GC disk is as easy as applying a couple of drops with 3.6  $\mu\text{L}$  of HRP solution, and letting it cure overnight [2].

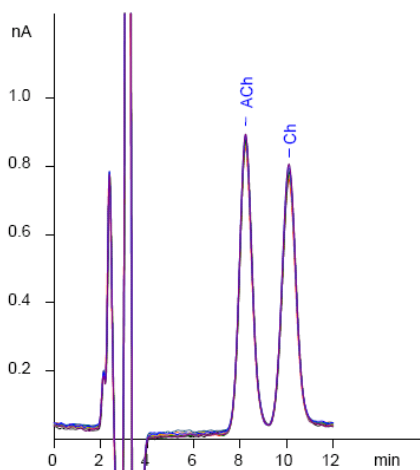


Fig. 2. Overlay of 10 chromatograms of 100 nM ACh and Ch in water. Injection volume 5  $\mu\text{L}$ ..

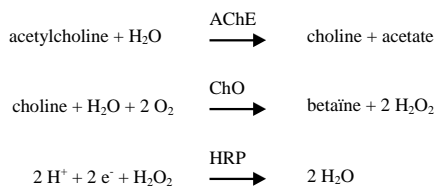


Fig. 3. Enzymatic conversion of acetylcholine and electrochemical detection of hydrogen peroxide on Pt or HRP-coated GC.

Table 1

Conditions	
HPLC	ALEXYS Acetylcholine Analyzer
Oven temperature	35 °C (separation and detection)
Flow rate	130 $\mu\text{L}/\text{min}$
Flow cell	Flexcell with HRP-coated GC
ADF™	0.01 Hz

### Reproducibility

The intra-day reproducibility was evaluated for standards of 100 nmol/L ACh and Ch in water (Fig. 2) and 50 nmol/L ACh in water in presence of 1  $\mu\text{M}$  Ch. A RSD for peak height is better than 2.2%, and for retention time RSD is 0.1%.

Inter-day repeatability was studied over 10 days. Based on 148 injections, peak heights have an RSD of about 15%, whereas retention times have 1% RSD.

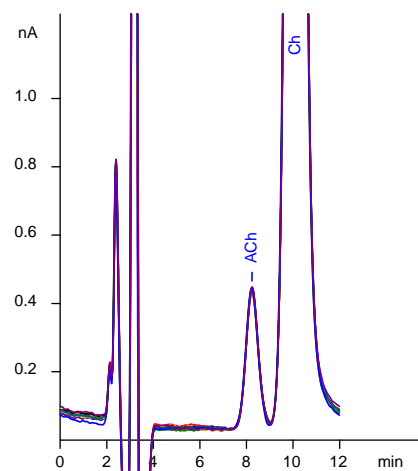


Fig. 4. Overlay of 10 chromatograms of 50 nM ACh in water with 1  $\mu\text{M}$  Ch. Injection volume 5  $\mu\text{L}$ ..

Table 2

Reproducibility (n=10)				
	Height, nA		Area, nA*sec	
	Mean	%RSD	mean	%RSD
50 nM ( in presence of 1 $\mu\text{M}$ Ch)				
ACh	0.40	1.5	14.8	1.6
100 nM				
ACh	0.84	1.6	32.2	1.6
Ch	0.74	2.2	31.1	2.3

## Linearity

The linearity of the method was determined in the concentration range of 20-100 nM for ACh and Ch. The method shows a good linear detector response with correlation coefficients of 0.998 or better (Table 3).

Linear regression curve ( $Y = aX + b$ )			
	Peak Height		
	a	b	r
ACh	1.71	-5	0.9994
Ch	1.50	-5	0.9979

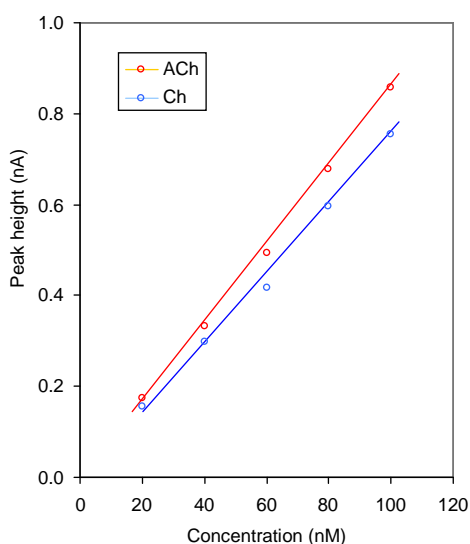


Fig. 5. Calibration plot for ACh and Ch (5 µL injections).

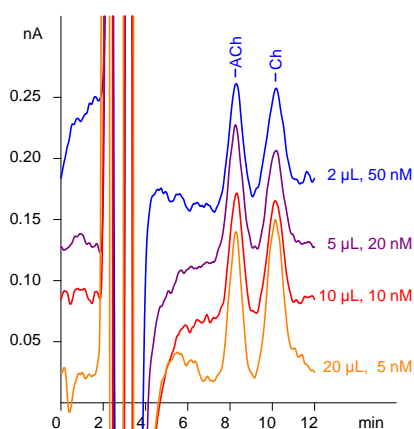


Fig. 6. Overlay of chromatograms of different combinations of concentration and injection volume that result in an on column load of 100 fmol.

## Detection limits and loadability

Detection limits were calculated as the concentration resulting in a signal that is 3 times the peak-to-peak noise of the baseline. Using the ALEXYS system in combination with the ACh/Ch assay kit and peroxidase electrode kit a Limit of Detection of 8 fmol on column for ACh and Ch could be reached. This corresponds with about 2 nM ACh and Ch on the basis of 5 µL injections and is comparable to the values as given in the kit-manuals.

If sample volume is not limited, even 20 µL sample can be injected without notable peak broadening and lower plate numbers. This would result in a factor 4 lower concentration detection limit of 0.5 nM (Fig. 6).

## Pt working electrode

Apart from the HRP-coated glassy carbon working electrode, H<sub>2</sub>O<sub>2</sub> resulting from the conversion of ACh and Ch can also be detected on Pt electrode in oxidative mode (polarity of detector is set to 'positive' in this case). Detection on Pt is somewhat less sensitive: about 80% relative response was found when comparing the two electrodes (Fig. 7). Other issues with the Pt electrode are that it takes longer for the baseline to stabilise, and it needs regular polishing. This electrode may be an acceptable alternative if enzyme stability during transport is an issue. The HRP enzyme has to be kept cold during transport, which can take too long for some parts of the world. Also, Pt can be used to trouble shoot the system. Reproducibility of results measured on a Pt electrode is comparable.

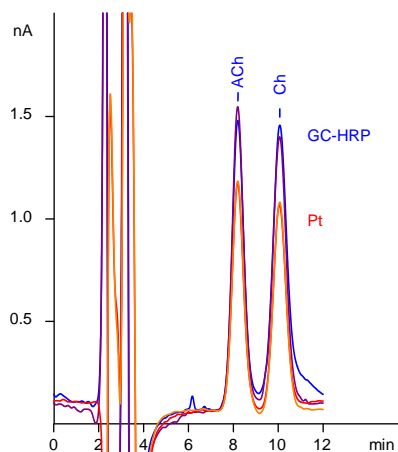


Fig. 7. Analysis of 100 nM ACh and Ch (5 µL) with GC-HRP electrode or Pt electrode.

## CONCLUSION

The ALEXYS Acetylcholine analyzer is a dedicated solution for the analysis of ACh and Ch. In combination with the BASi ACh/Ch assay kit and BASi peroxidase electrode kit sensitive, linear, and reproducible detection of ACh and Ch can be achieved.

## References

1. Liquid chromatographic methods used for microdialysis: an overview, SOPHIE SARRE, YVETTE MICHOTTE; Reference: from B.H.C. Westerink and T.I.F.H. Cremers (Eds.) Handbook of Microdialysis, vol. 16, 2007 Elsevier B.V.,
2. BASi user manual Acetylcholine kit (MF-8908).



Fig. 8. ALEXYS ACh and Ch Analyzer.

PART NUMBERS AND CONFIGURATIONS	
180.0067B	ALEXYS Acetylcholine analyzer
MF-8908*	BASi ACh/Ch assay kit incl column and IMER
MF-2096*	BASi Peroxidase refill kit
250.1700	In-line filter (aqueous)

\* The ALEXYS Acetylcholine analyzer is delivered including two BASi starter kits MF-8908 and MF-2096. Replacement kits and individual spare parts should be order from BASi directly. For more information see the BASi web site:

<http://www.basinc.com/products/ordering.html>