Application Note

Neuroscience

ALEXYS Analyzer for Highest Sensitivity in Neurotransmitter Analysis

Monoamines and Metabolites
Noradrenaline
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
Histamine (LNAAs)
4-aminobutyrate (GABA)
Glutamate (Glu)
LNAAs

Choline and Acetylcholine
Choline (Ch)
Acetylcholine (ACh)

Markers for oxidative stress
3-nitro-L-Tyrosine
8-OH-DPAT

Glutathione and other thiols

ALEXYS Neurotransmitter Analyzer

- **UHPLC-ECD**
- **Fast and sensitive**
- **Minimized sample use**
- **Analysis of monoamines, metabolites, GABA, Glu, ACh and Ch**

Summary

The ALEXYS Neurotransmitter Analyzer is a UHPLC-ECD system that has been developed for the analysis of a number of well-known neurotransmitters and metabolites. Dedicated and optimized methods are available and based on ECD and sub-2 µm packed columns, resulting in high plate numbers, short retention times and high detection sensitivity. For various monoamines, the detection limits are down to the lower pmol/L range. Where pre-column OPA-derivatization is involved, the LOD is around 10 nmol/L (GABA). In case the method involves in-line enzymatic conversion, the LOD is around 0.5 nmol/L (acetylcholine).

Introduction

The ALEXYS Neurotransmitter Analyzer (Fig. 1) consists of the P6.1L pump(s) with integrated degasser, the AS 110S autosampler, the DECADE Elite EC detector with SenCell flow cell and Clarity data acquisition software. It is a UHPLC-ECD system that has been optimized in all its details to benefit from the use of separation columns with sub-2 µm particles.

Figure 1: ALEXYS Neurotransmitter Analyzer

According to the Van Deemter equation, the sub-2 µm stationary phase particles not only result in a significant gain in efficiency (in comparison with the use of more traditional larger sized particles), but the efficiency does not diminish at increased flow rates or linear velocities (Fig. 2). The use of columns with sub-2 µm particles is therefore offering new possibilities to further improve neurotransmitter analyses with respect to separation efficiency, retention time and detection sensitivity.

UHPLC methods

UHPLC is characterized by extremely efficient separations in a short period of time and plate numbers of more than 100,000/m can be obtained using sub-2 µm particles. As a consequence, every detail in the flow path has to be optimized as it can become a bottle neck in getting the best possible separation performance. The peak efficiency or plate number N is described as the square of the retention volume (V_R) divided by the peak dispersion σ.

$$N = [\, V_R / \sigma \,]^2$$

Typically, tubing dimensions need to be smaller than 100 µm ID (Fig. 3), and loadability (injection volume) is limited (Fig. 4).

Peak broadening has been described [1 - 3] as the sum of all individual dispersion contributions (σ_{tot}) from injection (σ_{inj}), tubing (σ_{tub}), column (σ_{col}) and detection (σ_{det}):

$$\sigma_{tot}^2 = \sigma_{col}^2 + \sigma_{inj}^2 + \sigma_{tub}^2 + \sigma_{det}^2$$

To fully benefit from the improved column efficiency the other factors (injection, tubing, detection) must keep up to the same level. The dispersion from tubing is given as

$$\sigma_{tub}^2 = c\, L\, F\, d^4$$

where L is length and d is diameter of tubing, F is flow rate, and the constant c is π / 384 D (D is the diffusion coefficient).

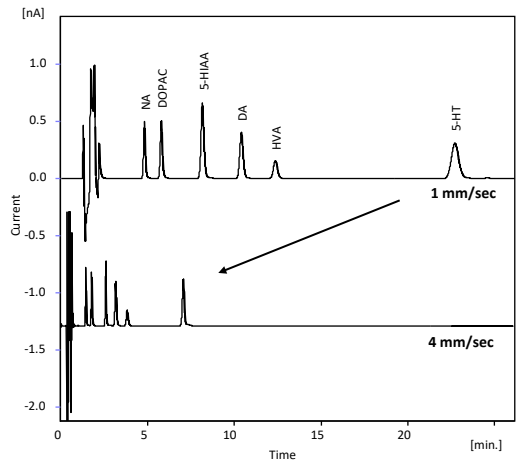


Figure 2: Fast separation of neurotransmitters with 80,000 -140,000 tp/m.

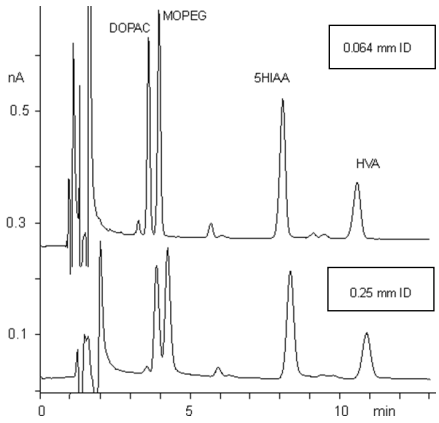
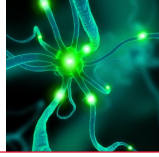


Figure 3: Dispersion by tubing. 64 µm vs 250 µm ID.



Dispersion by injection is directly proportional to the square of injection volume, and the same holds for detection in relation to detection cell volume

$$\sigma_{inj}^2 = a\, V_{inj}^2$$
$$\sigma_{det}^2 = b\, V_{det}^2$$

where *a* and *b* are constants.

A few practical implications can be deducted from this:

- *System pressure* - An increased flow rate in combination with small tubing diameter and a small particle size of stationary phase will contribute considerably to the system pressure. Therefore, the pump, injection valve, all tubing, nuts and ferrules must be rated for high pressure. Where possible the mobile phase is prepared with acetonitrile instead of methanol as this has a lower viscosity (smaller contribution to the total back pressure). An elevated temperature (35 – 45 °C) is sometimes applied for the same reason.

- *Response* - The fast separation and high plate numbers will have an impact on the signal. The peak height improves with the square root of the plate number. However, the loadability is limited to a few microliters to maintain the high plate number. Larger injection volumes can only be applied under stacking conditions without losing a significant number of plates.

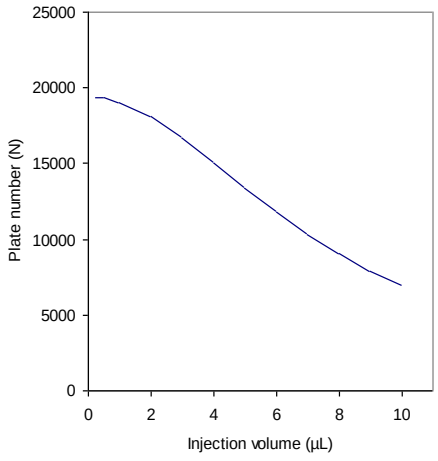


Figure 4: Injection volume affects plate count. Plot shows theoretical relationship; for peak eluting at 2 min at a flow rate of 150 uL/min on a column with 20,000 plates.

The use of a low pass noise filter is limited. By design, low pass frequency filters will attenuate high frequency signals including narrow peaks. As a rule of thumb the maximum allowed filter setting is related to retention time and inversely related to the square root of N

$$\tau < c\, t_R/\sqrt{N}$$

where c is 0.32 for max 10% loss in plate number.

As an example consider a regular HPLC peak with 4,000 plates at 10 min retention time: the application of a time constant of 3 sec is allowed. For a peak with the same plate number but 1.0 min retention time (UHPLC), the maximum time constant is 0.3 sec. Using a stronger filter setting would lead to significant loss of signal. Traditional noise filters will thus be useless in case of very fast separations. Moreover, very fast peaks require a high data acquisition frequency, which usually increases the noise level. It may be clear that there is a trade off between detection limit and speed of analysis, which may require a compromise in some cases.

Sample matrix conditions

Brain microdialysis is a common technique to sample neurotransmitters for analysis [4]. Microdialysate samples are 100% aqueous with a high concentration of salts (~150 mM). Collection and storage of samples from microdialysis usually requires precautions to avoid auto oxidation and degradation of substances of interest (e.g. catecholamines are prone to oxidation). Several authors report that their anti-oxidant mix contain either formic acid, or acetic acid or citric acid in combination with ascorbic acid or another anti-oxidant [5, 6]. This acidic/salty sample matrix has a considerable impact on the system peaks close to the solvent front and elsewhere in the chromatogram. These are a few recommendations to consider:

- In some cases, the presence of interfering peaks can be manipulated by modifying the octane sulfonic acid (OSA) concentration of the mobile phase (Fig. 5).

- To improve detection of peaks close to the solvent front, as a rule of thumb acidified samples should not contain more than 10 mM acid (end concentration).

- The salt bridge is to be preferred over the ISAAC (In Situ Ag/AgCl) reference electrode when peaks of interest are

eluting close to the front. When using the salt bridge, the baseline recovers more quickly from the initial destabilization (Fig. 6).

- The injection volume has an effect: smaller is better.

Optimization of separation conditions

Most neurotransmitters are very polar, and ion pairing chromatography on a (dedicated) C18 column is the separation method of choice in most cases. Temperature, ion pair concentration, the organic content and pH of the mobile phase are factors that can be adjusted to optimize the separation:

- *pH* - Retention of acidic metabolites (e.g. HVA, 5-HIAA, DOPAC) is selectively affected by pH on a C18 column: retention increases with lower pH due to the protonation of the carboxyl group in these components. The retention time of the derivatized amino acids (Glu, Asp, Gly etc) also responds to pH, but the response is component dependent.

- *Ion pair* - The retention of amines such as dopamine, noradrenaline, serotonin, choline and acetylcholine selectively increases with the ion pair concentration.

- *Temperature* - Higher temperatures results in faster elution. Separation between peaks can also be affected, as for instance the retention of serotonin is changed more significantly than that of the other components (Fig. 7).

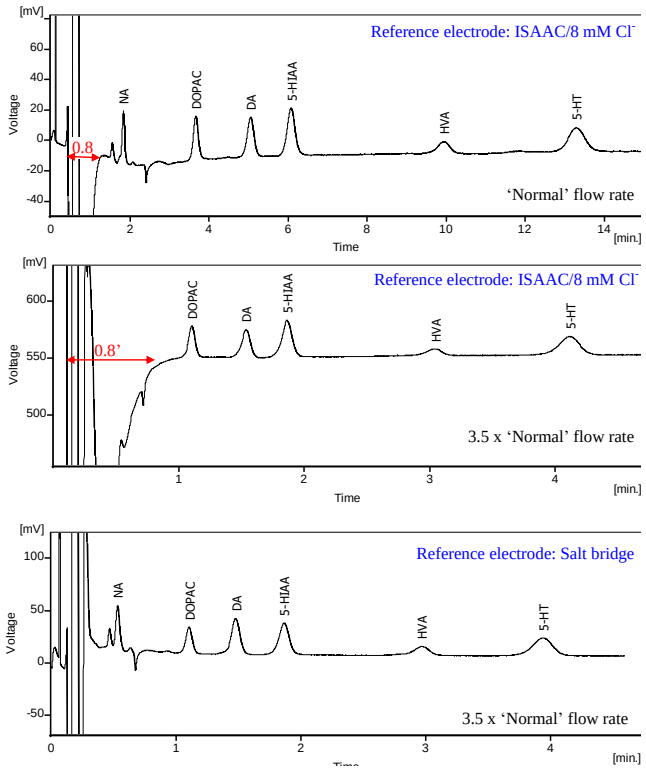


Figure 6: Analysis of 10 nmol/L standards in Ringer with 10 mM HAc. In case of ISAAC the NA peak disappears in the front with increased flow rate.

Optimization of separation conditions can be a time consuming part of method development. Antec Scientific's developed dedicated and optimized methods, described in separate application notes [7 - 9]; chromatograms of real samples may show interfering peaks, requiring further optimization.

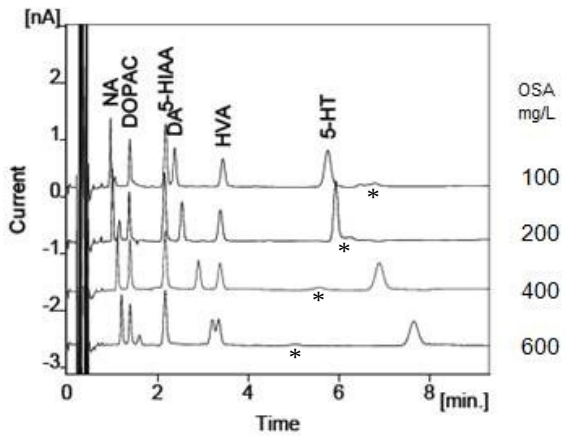


Figure 5: Effect of octane sulfonic acid (OSA) concentration in the mobile phase on neurotransmitters and baseline disturbance (*) elution.

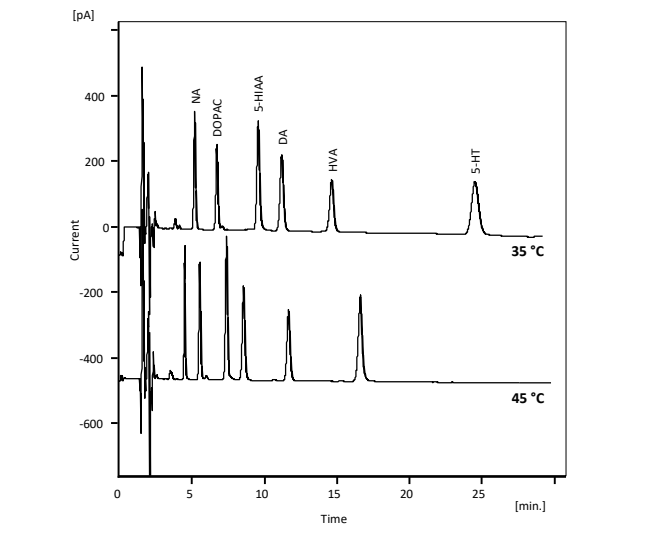


Figure 7: Effect of column temperature on separation.

Monoamines (NA, DA, 5-HT), metabolites

For simultaneous analysis of noradrenaline, dopamine and serotonin in microdialysis samples a fast UHPLC method has been developed. One of the challenges was to separate NA from the front peak and keep 5-HT within reasonable retention time.

LOD (s/n=3) of NA, DA and 5-HT is around 100 pmol/L. Linearity shows a correlation coefficient better than 0.999 in the range of 0.1 to 10 nmol/L. The relative standard deviation (RSD) is better than 2% for mid-range concentrations (5 nmol/L).

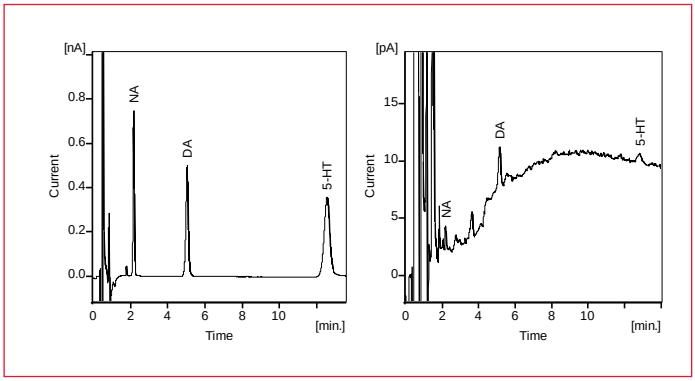


Figure 8: On the left analysis of 100 nmol/L standards in acidified Ringer. On the right a real sample: microdialysate of rat brain.

An example chromatogram showing the separation of 16 neurotransmitter, metabolites and related compounds is given in Fig. 9. For more details and variations of this analysis we refer to the Antec Scientific Application note [7].

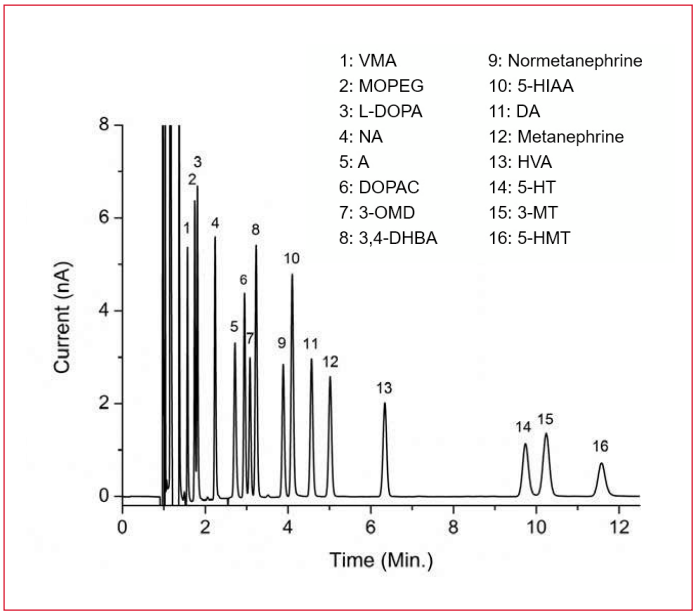


Figure 9: Standard mixture of 100 nmol/L neurotransmitters and metabolites.

GABA and glutamate

The analysis of GABA and glutamate is based on an automated in-needle derivatization routine with OPA reagent, followed by a fast UHPLC separation. A short post-analysis step gradient is applied to flush late eluting peaks off the column.

Detection limit of GABA and Glu is around 10 nmol/L (s/n=3). The linearity of the method was determined in the concentration ranges of 0.5 - 2.5 µmol/L Glu and 100 - 500 nmol/L GABA. The method showed a good linear detector response with correlation coefficients better than 0.998.

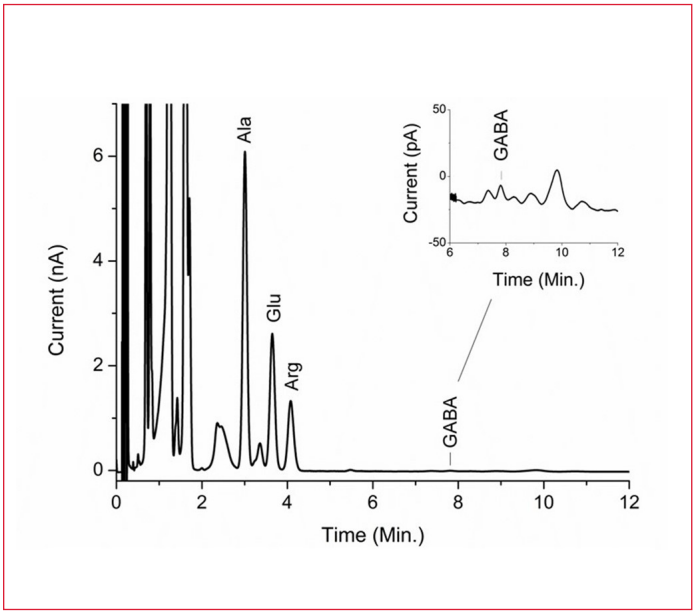


Figure 10: Example chromatogram of the analysis of GABA and Glu in a rat prefrontal cortex

In principle, all amino acids can be detected with this method [11 - 13]. With some modification to the separation parameters, this method can also be applied to detect other combinations.

For more details and variations of this analysis we refer to the Antec Scientific Application note [8]

Acetylcholine and choline

For the analysis of acetylcholine, a reversed phase ion-pairing separation method was evaluated. The method has a few interesting features compared to the alternative ion-exchange separation method.

The use of an analytical micro-bore UHPLC C18 column allows for faster separations. The a mobile phase with pH 7.5 improves lifetime of the IMER. The combination of UHPLC separation with optimal ECD working potential gives much better signal-to-noise ratio.

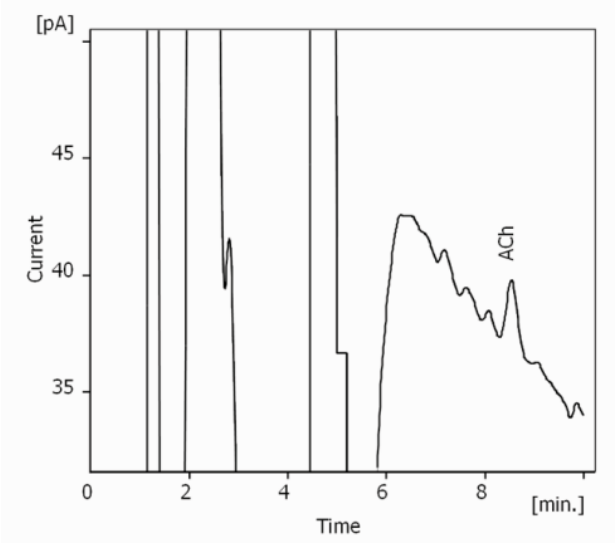


Figure 11: Chromatogram of 0.5 nmol/L acetylcholine in Ringer solution. This concentration is near the LOD.

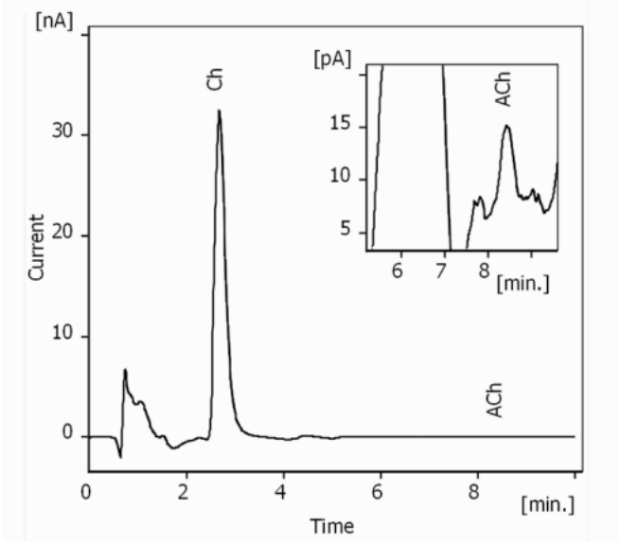
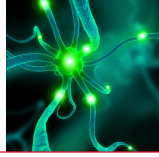


Figure 12: Chromatogram of a basal level rat microdialysate sample.

As acetylcholine is not readily detectable, it is first enzymatically converted to the electrochemically detectable hydrogen peroxide. This conversion takes place in a post column AChE/ ChOx immobilized enzyme reactor (IMER).

For detection, a platinum working electrode must be activated and after a short stabilization time the electrode is ready for use. Detection limit of acetylcholine is around 0.5 nmol/L (SN ratio = 3). Linearity shows a correlation coefficient better than 0.998 in the tested range of 0.5 - 20 nmol/L. The relative standard deviation (RSD) is better than 4% (tested with 10 nmol/L).

For more details of this analysis we refer to the Antec Scientific Application note [9].

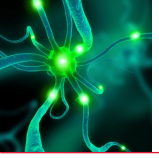


References

1. Šlais, K. and Kouřilová, D., 1983. Minimization of extra-column effects with microbore columns using electrochemical detection. *Journal of Chromatography A, 258*, pp 57-63.
2. Šlais, K, 1986. Small-Bore LC/EC. *Journal of chromatographic science, 24*(8), pp 321-329.
3. Prüß, A., Kempter, C., Gysler, J. and Jira, T., 2003. Extracolumn band broadening in capillary liquid chromatography. *Journal of Chromatography A, 1016*(2), pp.129-141.
4. Westerink, B.H. and Cremers, T.I., 2007. *Handbook of microdialysis: methods, applications and perspectives.* Elsevier.
5. Thorré, K., Pravda, M., Sarre, S., Ebinger, G. and Michotte, Y., 1997. New antioxidant mixture for long term stability of serotonin, dopamine and their metabolites in automated microbore liquid chromatography with dual electrochemical detection. *Journal of Chromatography B: Biomedical Sciences and Applications, 694*(2), pp.297-303.
6. Bicker, J., Fortuna, A., Alves, G. and Falcao, A., 2013. Liquid chromatographic methods for the quantification of catecholamines and their metabolites in several biological samples—a review. *Analytica chimica acta, 768*, pp.12-34.
7. Antec Scientific, Monoamines and acidic metabolites, Application note, 213.028
8. Antec Scientific, GABA and glutamate, Application note, 213.020
9. Antec Scientific, Acetylcholine and choline, Application note, 213.023
10. Nguyen, A.T., Aerts, T., Van Dam, D. and De Deyn, P.P., 2010. Biogenic amines and their metabolites in mouse brain tissue: Development, optimization and validation of an analytical HPLC method. *Journal of Chromatography B, 878* (29), pp.3003-3014.
11. Rowley, H.L., Martin, K.F. and Marsden, C.A., 1995. Determination of in vivo amino acid neurotransmitters by high-performance liquid chromatography with o-phthalaldehyde-sulphite derivatisation. *Journal of neuroscience methods, 57*(1), pp.93-99.
12. Jacobs, W.A., 1987. o-Phthalaldehyde-sulfite derivatisation of primary amines for liquid chromatography - electrochemistry. *Journal of Chromatography A, 392*, pp.435-441.
13. Smith, S. and Sharp, T., 1994. Measurement of GABA in rat brain microdialysates using o-phthaldialdehyde—sulphite derivatization and high-performance liquid chromatography with electrochemical detection. *Journal of Chromatography B: Biomedical Sciences and Applications, 652*(2), pp.228-233.
14. De Bundel, D., Sarre, S., Van Eeckhaut, A., Smolders, I. and Michotte, Y., 2008. Critical evaluation of acetylcholine determination in rat brain microdialysates using ion-pair liquid chromatography with amperometric detection. *Sensors, 8*(8), pp.5171-5185.

Conclusion

Fast and sensitive analysis of a number of neurotransmitters has been demonstrated using the ALEXYS Neurotransmitter Analyzer. It is a modular system with optimized methods for the most common neurotransmitters.



Ordering information

ALEXYS Neurotransmitter Analyzer for analysis of monoamines	
180.0091UW	ALEXYS Neurotransmitters SCC base
116.4120	SenCell with 2 mm GC WE and sb REF
250.1165*	Acquity UPLC in-line filter kit + 6 frits (205000343)
250.1166*	Acquity UPLC BEH C18, 1.7µm,1 x 50 mm (186002344)
250.1163*	Acquity UPLC BEH C18, 1.7µm,1 x 100 mm (186002346)

ALEXYS Neurotransmitter Analyzer for analysis of GABA and Glu	
180.0091UW	ALEXYS Neurotransmitters SCC base
180.0504W	Add-on parts for (2-pump) HPG option
116.4120	SenCell with 2 mm GC WE and sb REF
250.1160*	Acquity UPLC C18 HSS T3, 1x50 mm 1.8 µm (186003535)
250.1165*	Acquity UHPLC in-line filter kit + 6 frits (205000343)

ALEXYS Neurotransmitter Analyzer for analysis of ACh and Ch	
180.0091UW	ALEXYS Neurotransmitters SCC base
180.0505W	Add-on parts for ACh/Ch analysis
116.4322	SenCell 2 mm Pt HyREF
250.1165*	Acquity UPLC in-line filter kit + 6 frits (205000343)
250.1160*	Acquity UPLC C18 HSS T3, 1x50 mm 1.8 µm (186003535)
250.3532	AChE/ChOx IMER, 1mm

*) Columns are products of Waters Corporation (Milford, USA). The Waters part numbers are given between parenthesis for reordering purposes.

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