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Neurophysiologic detector—a selective and sensitive tool in high-performance liquid chromatography☆

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Abstract

In the present study neurons from the olfactory system of the fish crucian carp, *Carassius carassius* L. were used as components in an in-line neurophysiologic detector (NPD) to measure physiological activities following the separation of substances by high-performance liquid chromatography (HPLC). The skin of crucian carp, *C. carassius* L. contains pheromones that induce an alarm reaction in conspecifics. Extra-cellular recordings were made from neurons situated in the posterior part of the medial region of the olfactory bulb known to mediate this alarm reaction. The nervous activity of these specific neurons in the olfactory bulb of crucian carp was used as an in-line neurophysiologic detector. HPLC was performed with an HP 1100 model equipped with a diode array detector (DAD) and ChemStation software. An adsorbosphere nucleotide–nucleoside 7 μm column was used to separate the substances in the skin extract using artificial pound water (APW) as the mobile phase. UV spectral detection was performed at 214, 254 and 345 nm, and scans (190–400 nm) were collected continuously. This system enabled the selection of peaks in the chromatogram with fish alarm pheromone activity. The neurons in parts of the olfactory system from different aquatic organisms and vertebrates can be used for the detection of species-specific stimuli such as sexual and alarm signals, food odours, and other physiologically significant substances. NPDs clearly offer new and promising options for in-line HPLC as highly selective and sensitive detectors in biological, medical and pharmaceutical research.

Keywords: Neurophysiologic detector; Alarm pheromones

1. Introduction

This article is primarily concerned with the use of neurons from the olfactory epithelium and olfactory bulb of the fish *Carassius carassius* L. as a physiologically specific and sensitive detector for high-performance liquid chromatography (HPLC). By using the neurons from these parts of the nasal cavity and brain of the fish for detection, it was possible to recognize alarm pheromones in the chromatogram. Neurophysiologic detectors (NPDs) in-line with diode array detectors (DADs) are able to provide both the R_t of physiologically active substances and their spectral characteristics. In the paper only qualitative properties of the detector are discussed. The quantitative properties of NPDs are still under study. However, NPDs can be used successfully even at this stage for differentiation of minute amounts of alarm pheromones from mixtures of other substances.

From the very beginnings of chromatography [1,2] the importance of detection of the separated substances was evident. Without the possibility of detecting the substances of interest a chromatographic separation is meaningless. In the original experiments by Tswett in 1903, coloured components were detected visually. However, visual detection [1,2] is not always possible and other means of detection should be used [3]. Comprehensive reviews on the latest developments in detection techniques and detectors are regularly published [3,4]. Nearly all of the HPLC detectors used to date are bulk property or solute property detectors. These detectors lack the ability to identify substances with specific physiological activity passing through the detector cell. Notable exceptions include the electro-olfactogram (EOG) and electroantennogram (EAG) which are used with GC. Depolarization across an insect antenna in response to stimulation by volatile compounds was first shown by Schneider [5] in

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1957. In 1964 Ottoson and von Sydow were the first to describe the periodical exposure of frog olfactory membrane to gas flow from a GC for the separation of physiologically active substances [6]. In fact this was the first description of the use of physiologic detection methods in connection with GC. EAG in connection to GC is now a popular detection method for insect hormones, pheromones and repellents [7]. The electroantennographic detector was proposed in 1975 by Arn et al. [8]. The method described by Ottoson and von Sydow has not found many followers.

Von Frisch [9] realized that there were components in the fish skin in minnows (*Phoxinus phoxinus* L.) that induced alarm reactions in conspecifics. He showed that the reaction was elicited via the olfactory organ [10]. Despite several attempts to isolate and identify the alarm substances from *C. carassius* L., the identity of these pheromones is obscure [11–16].

In the crucian carp, *C. carassius* L., the secondary neurons of the posterior part of the medial olfactory bulb respond specifically to skin extracts [17]. These neurons supply the medial part of the medial olfactory tract (mMOT) that mediates the alarm reaction in crucian carp [18]. Because these neurones are selective and sensitive to the pheromones eliciting the alarm reaction, they can serve as detectors for these pheromones. The secondary neurons mediating the alarm reaction are situated in a restricted region of the olfactory bulb.

We have attempted to introduce in-line HPLC detectors based on neuro-electrochemical detection of physiologically significant substances. By this means we attempt to identify the R_t of substances of interest. In tandem with the NPD, the DAD provides the spectral characteristics of the alarm substance.

In the present study we made an in-line detection cell from the nasal cavity of crucian carp and the sensor unit from this particular region of the olfactory epithelium.

An olfactory bulb performed as the primary signal-filter and primary signal amplifier. The nasal cavity with olfactory epithelium and olfactory bulb played the role of the neurophysiologic detector.

2. Materials and methods

2.1. Preparation of neurophysiologic detector (NPD)

2.1.1. Biological material

Crucian carp, *C. carassius* L. (20–35 g) were caught in small lake (Tjernsrud) just outside Oslo city borders, Norway. They were transported to the aquaria facilities at the Department of Biology, University of Oslo where they were fed three times a week. Fish were initially anaesthetised with benzocaine (45 mg/l) and immobilised by intra-peritoneal injection of Saffan (Schering-Plough Animal Health, Welvyn Garden City, UK) 24 mg/kg body weight. To prevent drying and avoid any unforeseen movement during the experiment, fish were wrapped in a wet cloth and fixed by two steel rods, which fastened to upper parts of the orbital bones. Care was taken not to disrupt the tissue around the olfactory epithelium as damage to the skin causes release of alarm substances. Fish were continuously irrigated through the mouth and over the gills by pound water during the experiment. A schematic drawing of the experimental set-up of HPLC with NPD is shown in Fig. 1.

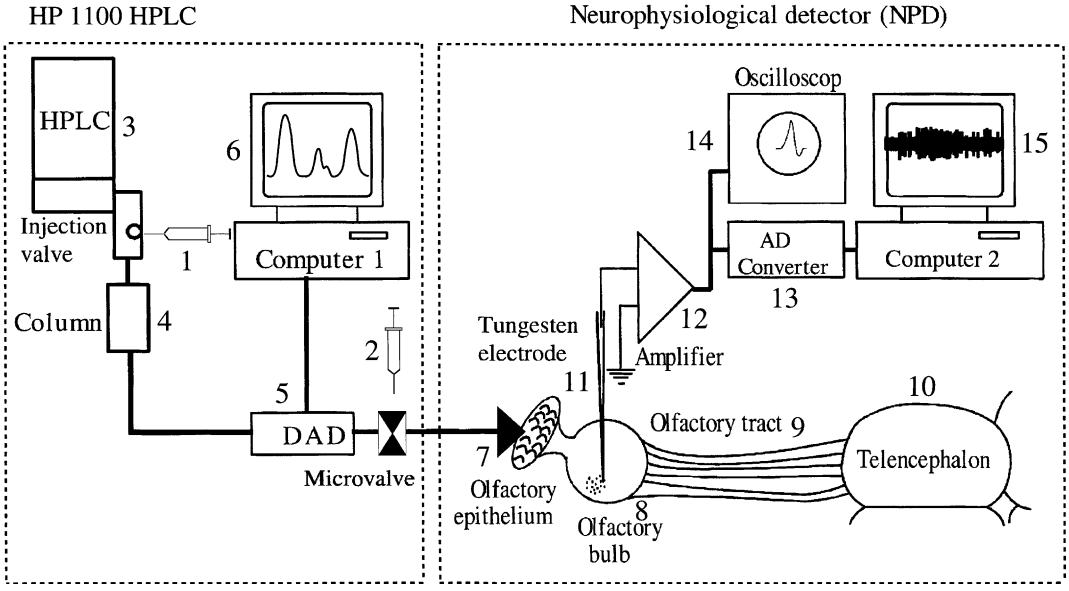

Fig. 1. A schematic drawing of the experimental set-up of HPLC with NPD: (1) analytical port; (2) calibration port; (3) HPLC pumps; (4) an adsorbosphere nucleotide–nucleoside 7 μm column; (5) a diode array detector; (6) a PC; (7) an olfactory epithelium; (8) an olfactory bulb; (9) olfactory tract; (10) a telencephalon; (11) a tungsten electrode; (12) an amplifier; (13) an AD converter; (14) an oscilloscope; (15) a PC. Details are described in the text.

2.1.2. Surgery

The skull above the olfactory tract and above the right olfactory bulb was removed under a stereo-microscope. The mesenchymeal tissue around the olfactory tract was aspirated by gentle sponging and the anterior part of the brain cavity was filled with paraffin oil.

The fish remained in good conditions for at least 8 h, as judged by the blood flow and the nervous activity recorded.

2.1.3. Conditioning

The olfactory organ was exposed to a continuous flow of artificial pound water (APW) NaCl (Merck, Darmstadt, Germany) 2.9×10^{-2} g/l, KCl (Merck) 3.7×10^{-3} g/l, $CaCl_2$ (Merck) 5.8×10^{-2} g/l, $NaHCO_3$ (Merck) 1.6×10^{-2} g/l.

The flow could be interrupted to give exposure to solutions of different compositions prepared in artificial pound water by a series of miniature valves or connected to the outlet from the DAD of the HPLC. The delay between the outlet from the DAD to the olfactory epithelium in nasal cavity of the experimental fish was found to be 3.4 s. The flow of the liquid through the nasal cavity was 0.5 ml/min.

2.1.4. Control of activity

The olfactory organ ipsilateral to the recording site (the right side) was exposed to diluted skin extracts 0.5 ml/min. When single unit activity at a recording site in the bulb was encountered that responded to this skin extract, the effluents from the HPLC 0.5 ml/min was switched on to the epithelium.

2.1.5. Preparation of fish skin extract (FSE) for control of activity

Crucian carps were killed by decapitation and skin was taken from the sides of the fish. Total weight of skin was approximately 2 g. The skin samples were placed in 100 ml distilled water and homogenised in a blander. The homogenate was filtered through glass wool and centrifugate with 6000 rpm 30 min. Supernatant was diluted with artificial pound water 1–10 and used for control of activity.

2.1.6. Preparation of fish skin extract (FSE) for HPLC

The skin extract was prepared as in Section 2.1.5. Supernatant 2 ml was subjected to hollow fibre filtration (HFF) using MicroKros (Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) column with 10 kDa cut off. Filtrate was used to chromatography.

2.1.7. Calibration and procedure

Extra-cellular recordings of signal from single (or few) neurons in the posterior part of the medial region of the olfactory bulb were performed with micro-electrodes made from tungsten wire (125 μm) prepared as described by Hubel [19]. Micro-electrode was advanced into the olfactory bulb.

The position of electrode was adjusted by an electrical micro-manipulator (SD instruments MC 1000, CA, USA). The signal was fed to an amplifier (Grass P55, Astro-Med Inc., Warwick, RI, USA) and the bandwidth adjusted to 0.3–3 kHz. A notch filter of 50 Hz was activated. The reference electrode was positioned on the border of the brain cavity. Signals from the amplifier were displayed on an oscilloscope (Tektronix 565, Portland, OR, USA). The nervous activity also was recorded and stored on a PC (Dell OptiPlex GX1p) via an AD converter (μ1401; CED, Cambridge, UK), and analysed with the aid of a software (CED. Spike 2, version 4.04, CED, Cambridge, UK).

The nervous activity observed when exposing the olfactory epithelium with APW as mobile phase passing through

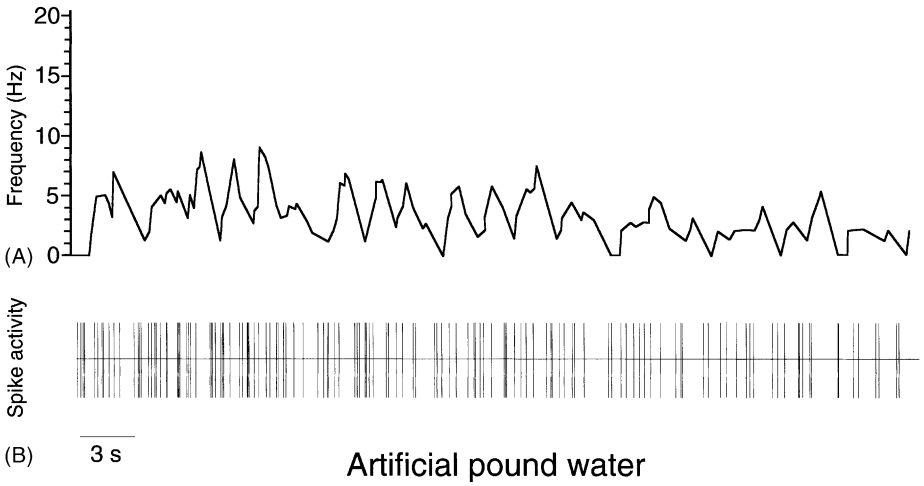

Fig. 2. The neurons signal activity was recorded for direct use of artificial pound water (APW) as the mobile phase passing through column. This neurons signal activity was accepted as a base line for NPD. (A) The corresponding mean low frequency of the spikes. (B) Spontaneous activity of single units recorded from the posterior part of the medial region of the olfactory bulb. Details are described in the text.

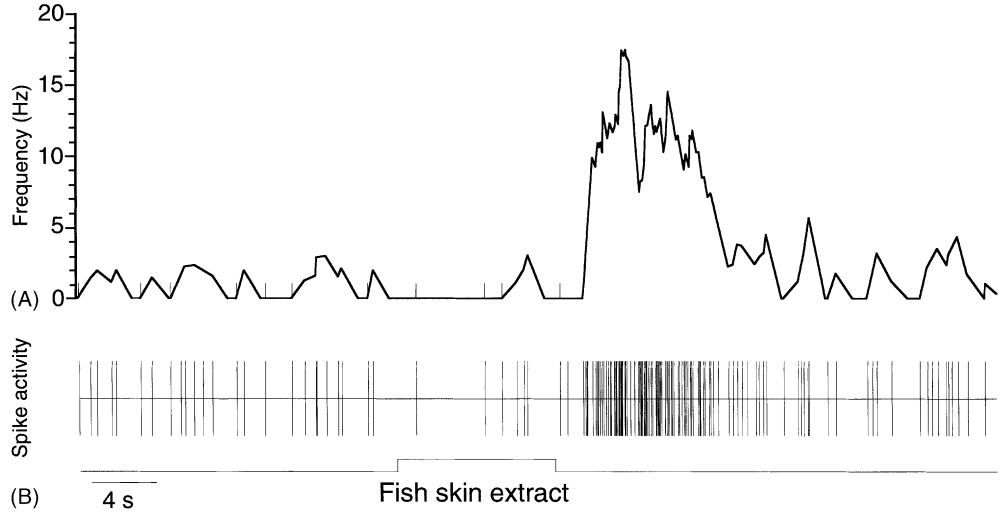

Fig. 3. The neurons signal activity due to fish skin extract (FSE) introduced in the nasal cavity by bypassing the column using the calibration port 2. This neurons signal activity served as a control and a reference activity. (A) The corresponding mean high frequency of the spikes. Note the increase in nervous activity seen in the mean frequency diagram. (B) Neurons activity of single units recorded from the posterior part of the medial region of the olfactory bulb. Details are described in the text.

column (Fig. 2) was accepted as a base line for NPD. The nervous activity observed when stimulating the olfactory epithelium with fish skin extract bypassing the column using the calibration port was accepted as a control and reference activity Fig. 3.

2.2. High-performance liquid chromatography (HPLC)

HPLC was performed with an HP 1100 model produced of Hewlett-Packard (Palo, Alto, CA, USA) equipped with a diode array detector, and ChemStation (Palo, Alto, CA, USA) software was used. Analysis were done on the adsorbosphere nucleotide–nucleoside 7 μm column 250 mm long and 4.6 mm i.d., from Alltech (Alltech Associates Inc., Deerfield, IL) with a flow of 0.5 ml/min. The mobile phase was artificial pond water APW. The injection volume was 200 μl. Detection was done at 214 and 254 nm, simultaneously UV scan was done in the range 190–400 nm.

The UV spectra were detected and collected in this range. NPD detector was connected to the DAD by the PTF tub-

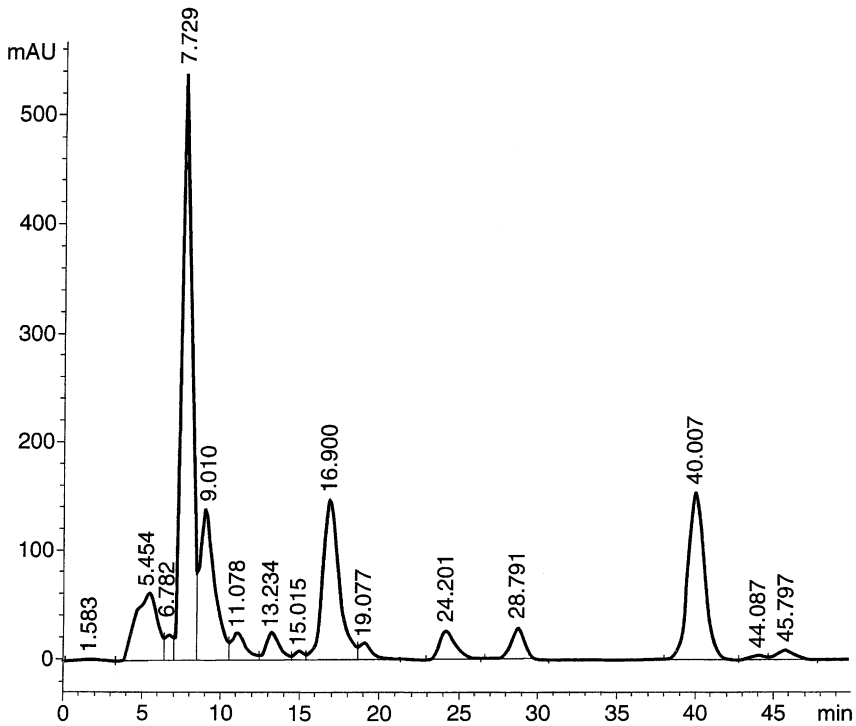

Fig. 4. A characteristic chromatogram of fish skin extract (FSE) with UV detection at 214 nm. Details are described in the text.

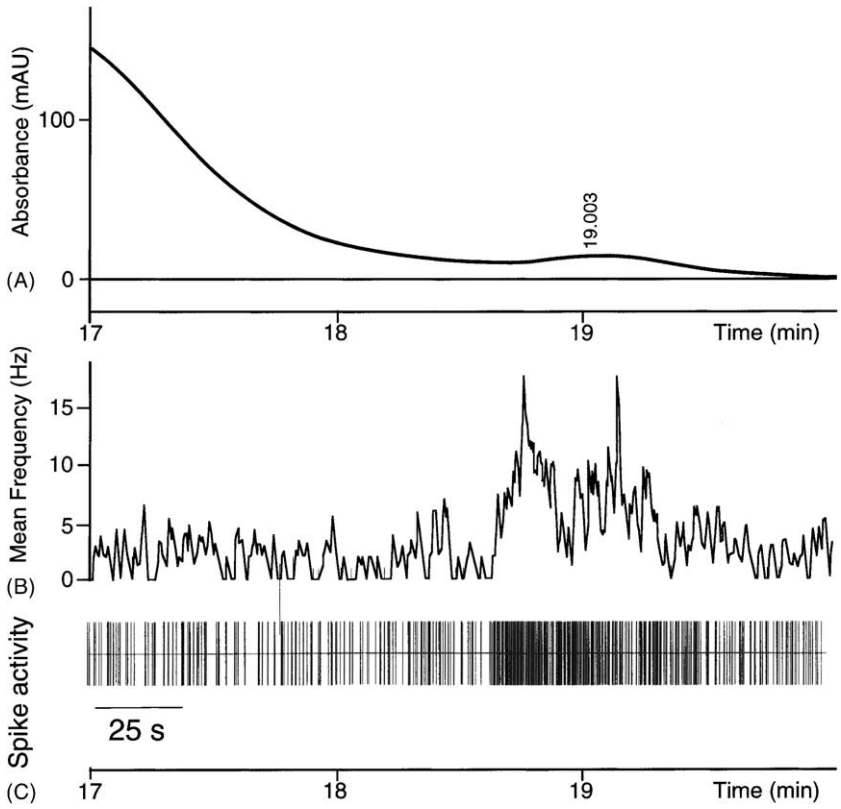

Fig. 5. An extended part (from 17 to 20 min) of the chromatogram in Fig. 4 with simultaneous recording of neurons activity. (A) The extended part of the chromatogram with active peak eluted at R_t 19.003 min. (B) The corresponding mean frequency of the nervous activity. Note the increase in mean frequency concomitant with the appearance of the peak at 19.003 min. (C) Neurons activity of single units recorded from the posterior part of the medial region of the olfactory bulb. Note the increase in the nervous activity concomitant with the appearance of the peak at 19.003 min. Details are described in the text.

ing 0.2 mm i.d. and 90 cm long (delay volume is 28.3 mm^3). Characteristic chromatogram shown in Fig. 4. the extended part of this chromatogram with simultaneous recording of neurons activity evoked by the putative alarm substance shown in Fig. 5.

2.2.1. Reprocessing of the HPLC separation

Reprocessing was done with instrumentation and condition as described above, with the change of the injection volume from 200 to 500 μl, chromatogram is not shown. 3D plot of active site was obtained in range 190–400 nm. 3D plot is not shown. Second reprocessing was done using UV detection at 345 nm and reference at 395 nm. A chromatogram is shown in Fig. 6.

3. Results

Sixteen series of experiments were run with the set-up displayed in Fig. 1. No difference in neurons signal activity was recorded when artificial pound water was used as the mobile phase passed through the column (Fig. 2). The neurones signal activity due to fish skin extract introduced in the nasal cavity by bypassing the column using the calibration port was recorded and served as a control and reference activity (Fig. 3).

By using artificial pound water was possible to elute the alarm substances from the column. After 19 min of elution time it was possible to detect nerve activity Fig. 5. This activity clearly corresponded with a peak recorded by the DAD. The other peak with R_t eluting at around 5 min evoked a moderate increased activity of neurons. This peak demonstrated a moderate increase in activity over a period of few seconds.

A 3D plot provided basis for optimized detection conditions as follows: detection at 345 nm and reference at 395 nm.

A characteristic chromatogram is shown in Fig. 6. The chromatogram consists of two peaks similar in spectral characteristics and physiological activity, at both peaks there was an increase in the nervous activity in the neurons of the olfactory bulb.

4. Discussion

The results of the present study demonstrate that it is possible to construct a detector cell and primer signal-filter from the nasal cavity, neurons from the olfactory epithelium

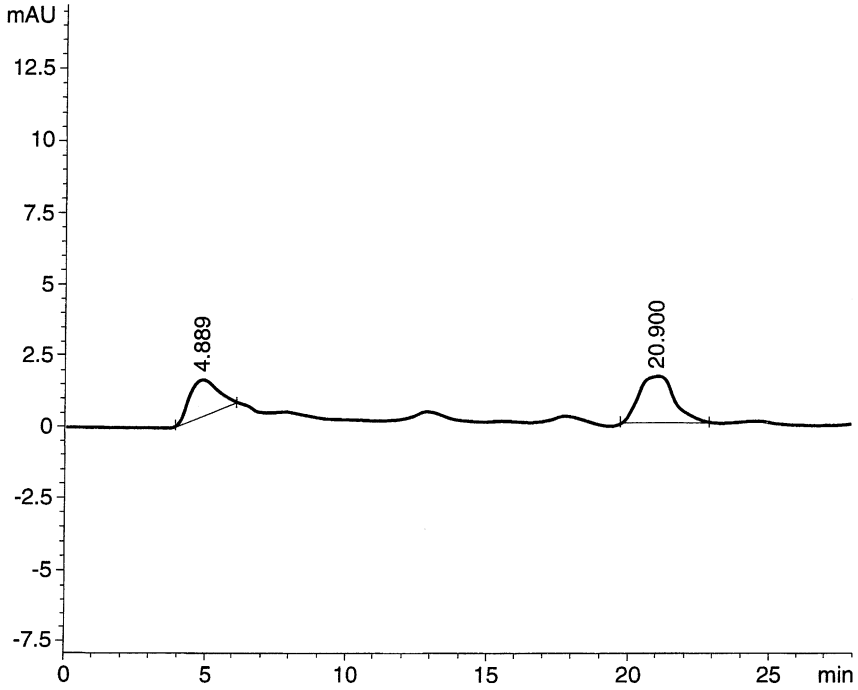

Fig. 6. Reprocessing of fish skin extract (FSE) separation using optimized UV detection at 345 nm and reference at 395 nm. The chromatogram consists of tow peaks similar in spectral characteristics and physiological activity. The peak eluted at R_t 4.889 and 20.900 min. Details are described in the text.

and olfactory bulb of living fish. This construction functions as a highly selective detection device. By using the present method, one takes advantage of the specific projection of the sensory neurons to specific region of the olfactory bulb [18,20,21]. In fish, the surface recordings from the olfactory bulb have shown that these are distinct zones that respond to particular odorants [22,23].

These results have been corroborated by recording the glomerular activity in the bulb [24,25] and by recording the single unit activity [17,26,27]. A strong argument for

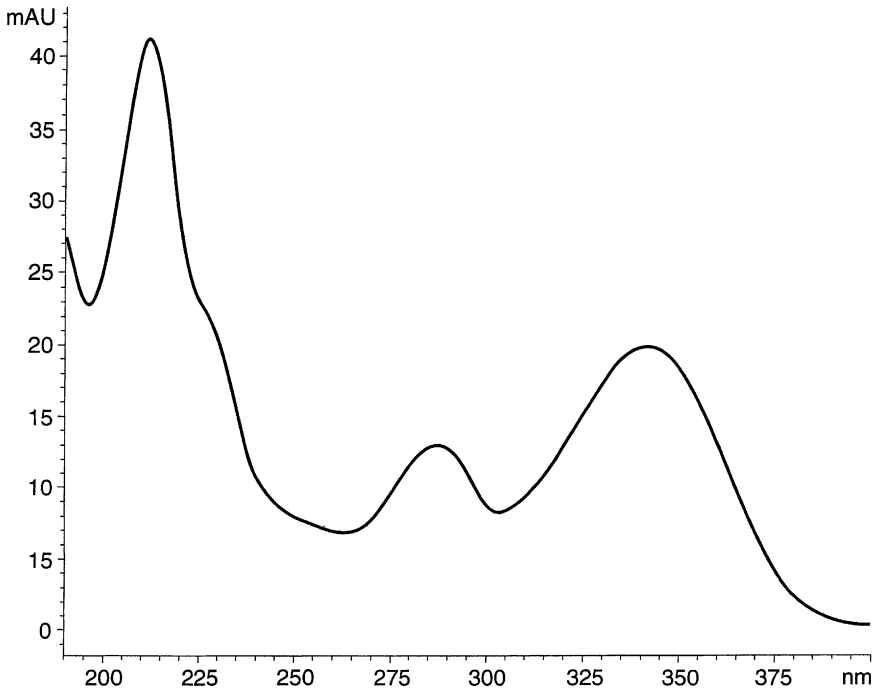

Fig. 7. The dynamically recorded UV spectrum of alarm substance from fish skin extract (FSE) of *C. carassius* L. Details are described in the text.

the specificity of the bulbar region is the fact that electrical stimulation of the different bundles of the olfactory tract induces different behaviours associated with feeding, reproduction and alarm [28,29]. The presented system enables the selection of peaks with fish alarm pheromones activity, with high reproducibility and selectivity. We have not quantified the sensitivity of the detector to the alarm substance because the authentic alarm substance for *C. carassius* L. has not yet been isolated. It has been possible in the first instance to demonstrate that the alarm reaction is evoked by a multi-component mixture. Using a DAD as a spectral detector in connection to the NPD gave the opportunity to do 3 D spectral analysis of active sites, reprocessing the HPLC separation to receive the refined chromatogram and its dynamically recorded UV spectrum as shown in Fig. 7. This spectrum is the documentation of the UV spectral characteristics of the alarm substance in *C. carassius* L. showing that the alarm substance is a highly unsaturated molecule, possible bicyclical and possible containing heteroatom.

5. Conclusion

5.1. Shortcomings

The detector cell has a short lifetime, and is operative for about 8 h.

5.2. Advantages

The present detector device offers new possibilities in analysing the relationship of substances to physiological effects directly during separation of complex mixtures by HPLC. The neurones from olfactory organs from different organisms could thus be used for the detection of species-specific stimuli such as sexual and alarm signals, food, and other physiologically significant substances. NPDs clearly offer new and promising options for in-line HPLC detection in biological, medical and pharmaceutical research.

5.3. Characterization of physiologically active substances

1. NPD gives the opportunity for the direct recognition and characterization of physiologically active substances under HPLC separation. It is an HPLC detector showing response to physiological activity.
2. NPD can be operated in connection with other detectors such as UV, NMR, etc.

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References

[1] M.S. Tswett, Otd. Biol. 14 (1903) 1 (in Russian).
[2] M.S. Tswett, Ber. Dtsch. Bot. Ges. 24 (1906) 384.
[3] I. Brondz, Anal. Chim. Acta 465 (2002) 1.
[4] R.P.W. Scott, Liquid Chromatography Detectors, Journal of Chromatography Library, vol. 33, Elsevier, Amsterdam, 1986.
[5] D. Schneider, Z. Vergl, Physiol. 40 (8) (1957) 41.
[6] D. Ottoson, E. von Sydow, Life Sci. 3 (1964) 1111.
[7] L.J. Wadhams, in: A.R. McCaffrey, I.D. Wilson (Eds.), Chromatography and isolation of insect hormones and pheromones, Plenum Press, New York, 1990, p. 289.
[8] H. Arn, E. Städler, S. Rauscher, Z. Naturforsch. 30 (1975) 722.
[9] K. von Frish, Naturwissenschaften 26 (1938) 601.
[10] K. von Frish, Z. Vergl. Physiol. 29 (1941) 46.
[11] R. Huttel, Naturwissenschaften 29 (1941) 333.
[12] W. Pfeiffer, J. Lemke, J. Comp. Physiol. 82 (1973) 407.
[13] M. Argentini, Isolerung des Schreckstoffes aus der Haut der Elritze “Phoxinux phoxinux L.” Philosophischen Fakultat II Zurich, Universitat Zurich, 1976, p. 111.
[14] A.O. Kasumyan, N.E. Lebedeva, J. Ichthyol. 19 (1979) 109.
[15] A.O. Kasumyan, V.Y. Ponomarev, J. Evol. Biochem. Physiol. 23 (1987) 20.
[16] T. Win, Isolation and structural identification of an alarm pheromone from the giant danio danio malabarcus (Cyprinidae, Ostariophysi, Pisces), Thesis, der Carl-von-Ossietzky University, Oldenburg, 2000.
[17] E.H. Hamdani, K.B. Døving, Chem. Senses 28 (2003) 181.
[18] K.J. Ressler, S.L. Sallivan, L.B. Buck, Cell 79 (1994) 1245.
[19] D.H. Hubel, Science 125 (1957) 549.
[20] R. Vassar, S.K. Chao, R. Sitcheran, J.M. Nunez, L.B. Vosshall, R. Axel, Cell 79 (1994) 881.
[21] P. Mombaerts, F. Wang, C. Dulac, S.K. Chao, A. Numes, M. Mendelson, J. Edmondson, R. Axel, Cell 87 (1996) 675.
[22] G. Thommesen, Acta Physiol. Scand. 102 (1978) 205.
[23] K.B. Døving, R. Selset, G. Thommesen, Acta Physiol. Scand. 108 (1980) 123.
[24] R.W. Friedrich, S.I. Korsching, Neuron 18 (1997) 737.
[25] R.W. Friedrich, S.I. Korsching, Neuroscience 18 (1998) 9977.
[26] P.W. Sorensen, Chem. Senses 21 (1996) 245.
[27] A.A. Nikonov, J. Caprio, J. Neurophysiol. 86 (2001) 1869.
[28] K.B. Døving, R. Selset, Science 207 (1980) 559.
[29] L.S. Demski, J.G. Dulka, Brain Res. 291 (1984) 241.