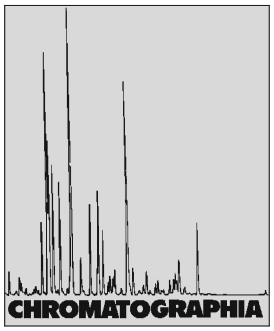
Enzymatic Reaction Coupled with Flow-Injection Analysis with Charged Aerosol, Coulometric, or Amperometric Detection for Estimation of Contamination of the Environment by Pesticides



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Abstract

Because excessive using of pesticides poses a threat to the environment and to human health, development of low-cost and sensitive methods for analysis of pesticides in the environment is needed. Several bacteria can release halide ions from the molecules of halogenated hydrocarbons. This can be used in a device for analysis of halogenated hydrocarbons in the environment by quantification of the halide anions. Here we directed our attention to selecting an instrument for detection of chloride anions. We tested three different detectors, amperometric, and coulometric, both coupled with flow-injection analysis and charged aerosol, coupled with high performance liquid chromatography. Detection limits ($3 \times S/N$) for measurement of chloride anions by use of these detectors was 30 μM (charged aerosol), 100 nM (coulometric), and 1 nM (amperometric). Because of its lowest detection limit for chloride anions and the many technical possibilities of miniaturization, the amperometric detector was used to test of effect of different cations on the chloride signal under the optimized experimental conditions (working electrode potential −365 mV; ''Current *R*'' 5 μA; mobile phase 0.2 M phosphate buffer, pH 6; flow rate 0.5 mL min^{-1}). NaCl, $SrCl_2$, NH_4Cl, and CsCl were tested as sources of chloride anions. We then used the detector to detect chloride anions catalytically cleaved from 1-chlorohexane by the enzyme haloalkane dehalogenase LinB from the bacterium *Sphingobium japonicum* UT26. The activity of the enzyme increased with increasing reaction temperature until the maximum was observed at 39°C. The results obtained were in good agreement with data obtained by colorimetric detection.

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Keywords

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Electrochemical detection
Charged aerosol detection
Enzyme
Environmental analysis
Alkyl halides
Chloride

Introduction

To ensure sufficient food supply for the world's increasing population a variety pest-control chemicals (''pesticides'') is used in agriculture. Organophosphate, carbamate, and organochlorinated compounds are the most widely used organic pesticides. The main attention of many scientists is directed toward chlorinated hydrocarbons, which affect the nervous and respiratory system and, unfortunately, persist in the environment. In developed countries their use is strictly regulated, and new technologies are sought to remove their residues [1–8]. In developing countries, however, there is no restriction on the use of these substances.

Monitoring of the environment for pollution by pesticides is, therefore, a concern in both developed and developing countries. Development of low-cost, sensitive methods for analysis of pesticides in the environment is needed [9–13]. For direct detection of these molecules hyphenated techniques, for example gas or liquid chromatography with mass spectrometry [14–21], and capillary electrophoresis [22, 23] or thin-layer chromatography [24] coupled with a variety of detectors, are used. These methods are often time-consuming, require high-cost instrumentation, cannot be miniaturized.

Enzymes called haloalkane dehalogenases have recently been discovered and isolated from, primarily, bacterial strains living in soil contaminated with halogenated aliphatic compounds [25–27]. The enzymes are involved in biochemical pathways enabling bacteria to utilize halogenated hydrocarbons by releasing halide ion from the molecule [27–31]. This property can be used in a device for analysis of chlorinated hydrocarbons in the environment by quantification of chloride anions. Methods used for quantification of chloride ions have been reviewed by Villagran et al. [32]; potentiometry [10, 33, 34], spectrometry [35, 36], conductometry [37], amperometry [38], and voltammetry [39] have been used.

In this work we directed our attention to selecting an instrument for detection of chloride ions. We tested three different detectors, the CoulochemIII amperometric detector and the CoulArray coulometric detector, both coupled with flow-injection analysis and the Corona charged-aerosol detector, coupled with high performance liquid chromatography, for detection of chloride ions. The most sensitive detector was used to study dechlorination of chlorinated hydrocarbons catalyzed by the enzyme haloalkane dehalogenase isolated from the bacterium *Sphingobium japonicum* UT26.

Experimental

Chemicals and Reagents

Chemicals of ACS purity were purchased from Sigma-Aldrich (St Louis, USA) unless indicated otherwise. Before analysis, all solutions were filtered through 0.45-μm Teflon membrane filters (Metachem, Torrance, CA, USA).

Instrumentation

High-performance liquid chromatography coupled with the corona charged-aerosol detector.

HPLC-CAD was performed with two model 582 solvent-delivery pumps, the Corona charged-aerosol detector (CAD), a nitrogen generator (all from ESA, Chelmsford, MA, USA), and a 150 × 4.1 mm, 10-μm particle, Hamilton (Reno, Nevada, USA) PRP-X100 column. The sample (50 μL) was injected by means of a Microtiter HPLC model 540 autosampler (ESA, USA). The detector and column temperatures were controlled with an ESA thermostatic module. Chromatograms were processed using Clarity ver. 2.4.1.93 (DataApex, Czech Republic).

Flow-Injection Analysis Coupled with the CoulArray Coulometric Detector

FIA-ED was performed with two model 582 solvent-delivery pumps (ESA), a reaction coil (1 m), and a CoulArray model 5600A electrochemical detector (ESA). The detector comprises three flow cells (model 6210; ESA), each consisting of four analytical cells containing a working porous carbon electrode, two auxiliary electrodes, and two reference electrodes. The detector and the reaction coil were thermostatted. The sample (10 μL) was injected by use of the Microtiter model 540 autosampler.

Flow-Injection Analysis Coupled with the CoulochemIII Amperometric Detector

FIA-AD was performed with a model 582 solvent-delivery pump (ESA), a model 5020 guard cell (ESA), a reaction coil (1 m), and the model 5040 amperometric detector (ESA). The detector comprises one low-volume flow-through analytical cell containing a glassy carbon working electrode, a hydrogen-palladium electrode as reference electrode, and an auxiliary carbon electrode, and the CoulochemIII control module. The sample (5 μL) was injected manually, at room temperature, by use of a dosing valve. The data obtained were processed by CSW 32 software. The FIA-AD conditions were: mobile phase 0.2 M phosphate buffer; potential of guard cell 0 mV. Mobile phase flow rate and pH, the potential of the working electrode, and ''Current *R*'' were optimized as

described in the "Results and Discussion" section.

Spectrophotometric Measurement

The effect of temperature on haloalkane dehalogenase activity was determined by performing activity assays at different temperatures from 20 to 50 °C. Dehalogenase activity was assayed by a colorimetric method developed by Iwasaki and co-workers [36]. Released halide ions were analyzed spectrophotometrically, at 460 nm, using a Sunrise (Tecan, Grödig/Salzburg, Austria) microplate reader, after reaction of the ions with mercuric thiocyanate and ferric ammonium sulphate. The dehalogenation reaction was performed in 25-mL Reacti Flasks closed by Mininert valves. The reaction mixture comprised 15 mL glycine buffer (pH 8.6) and 2 µL 1-halidehexane as substrate. The reaction was initiated by addition of enzyme at a final concentration 0.15 µM. The reaction was monitored by collecting of 1 mL of the mixture after reaction for 5, 10, 15, 20, 25, 30, 40, 50, and 60 min. After collection, reaction mixture samples were immediately mixed with 0.1 mL 35% nitric acid to terminate the reaction. Dehalogenation activity was quantified as the rate of production of halide ion.

pH Measurement

pH was measured with a WTW inoLab Level 3 with Level 3 terminal (MultiLab Pilot; Weilheim, Germany). The pH electrode (SenTix H) was calibrated by set of WTW buffers (Weilheim).

Preparation of Haloalkane Dehalogenase

To overproduce haloalkane dehalogenase, the corresponding gene was cloned in pAQN vector and transcribed by the *tac* promoter (P *tac*) under the control of *lacI*^{q}. *Escherichia coli* BL21 cells containing this plasmid were cultured in 0.25 L Luria broth at 37 °C. Enzyme expression was initiated by addition of isopropyl-*β*-D-thiogalactopyranoside at a final concentration of 0.5 mM when the culture reached an optical density of 0.6, measured at 600 nm. After induction, the culture was incubated at 30 °C for 4 h and then harvested. The cells were disrupted by sonication with a Soniprep 150 (Sanyo, UK). The supernatant was used after centrifugation for 1 h at 21,000*g*. The crude extract was further purified on a HiTrap Chelating column (Amersham Biosciences, Germany) charged with Ni^{2+}. The His-tagged dehalogenase was bound to the resin in equilibrating buffer containing 20 mM potassium phosphate buffer, pH 7.5, 0.5 M sodium chloride, and 10 mM imidazole. Unbound and weakly bound proteins were eluted with buffer containing 60 mM imidazole. The His-tagged enzyme was then eluted by buffer containing 250 mM imidazole. The active fractions were pooled and dialyzed overnight against 50 mM potassium phosphate buffer, pH 7.5. The enzyme was lyophilized and stored at 4 °C. Before use the enzyme was dissolved in ACS water at 6.4 mg mL^{-1}. 1-chlorohexane (98%) was used as substrate for the enzymatic reaction. To study temperature dependence, the enzyme (0.4 mg mL^{-1}) was incubated with the substrate (2 mM), in the presence of borate buffer (pH 7.55), at different temperatures, for 80 min (Thermomixer Eppendorf, 250 rpm).

Results and Discussion

Detection of Chloride Ions Using the Corona Detector

Instruments such as the Corona charged-aerosol detector (CAD) can be used to detect non-volatile compounds containing inorganic anions. Chloride, bromide, and nitrate anions (50 µL) were injected into the Corona system, with 60 mM ammonia and 100 mM formic acid at 2 mL min^{-1} as isocratic mobile phase. Detector sensitivity was set at 20 pA. Three signals corresponding to chloride, bromide, and nitrate were observed (Fig. 1). The detection limit for chloride ions, calculated, according to Long [40], as (3 × S/N), where *N* is the standard deviation of the noise determined in the region of the signal, was estimated as 30 µM. Other approaches for estimation of detection limits have been reported by Lavagnini et al. [41].

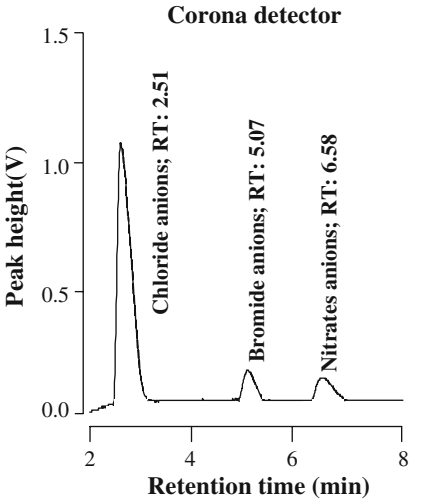

Fig. 1. The Corona detector. HPLC-CAD chromatogram obtained from chloride, bromide, and nitrate anions (1 mM)

Detection of Chloride Ions Using the CoulArray Detector

Chloride anions were also detected by use of the twelve-channel CoulArray detector. Detection potentials within the range −150 to −350 mV were applied to the electrodes to find the highest response to the target ion. The highest electrochemical response was observed at −250 mV. At higher potentials the signal obtained from the chloride standard (100 µM) decreased slightly (Fig. 2a). A potential of −250 mV was subsequently applied to all working electrodes and chloride anion peak height was measured (Fig. 2b). The chloride anion signal measured at the surface of the twelve electrodes gradually decreased. We first observed this phenomenon in the analysis of low-molecular-mass thiols [42] and have since published several papers describing the phenomenon [43–48]. This phenomenon is closely related to the serial connection of porous working electrodes. At the surface of the first electrode the highest signal is measured. The signal then decreases because of electrochemical conversion of part of the total amount of target molecule.

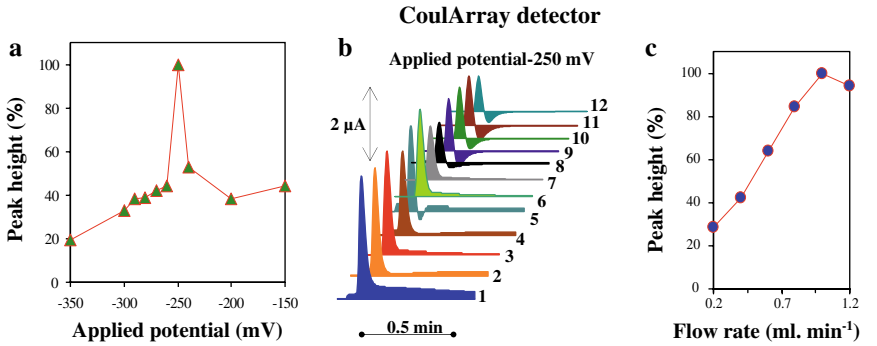

Fig. 2. The CoulArray detector. Effect of **a** applied potential and **c** mobile phase flow rate on response to chloride anions. **b** FIA-ED output at an applied potential of −250 mV measured at twelve working electrodes. The concentration of chloride anions was 100 µM

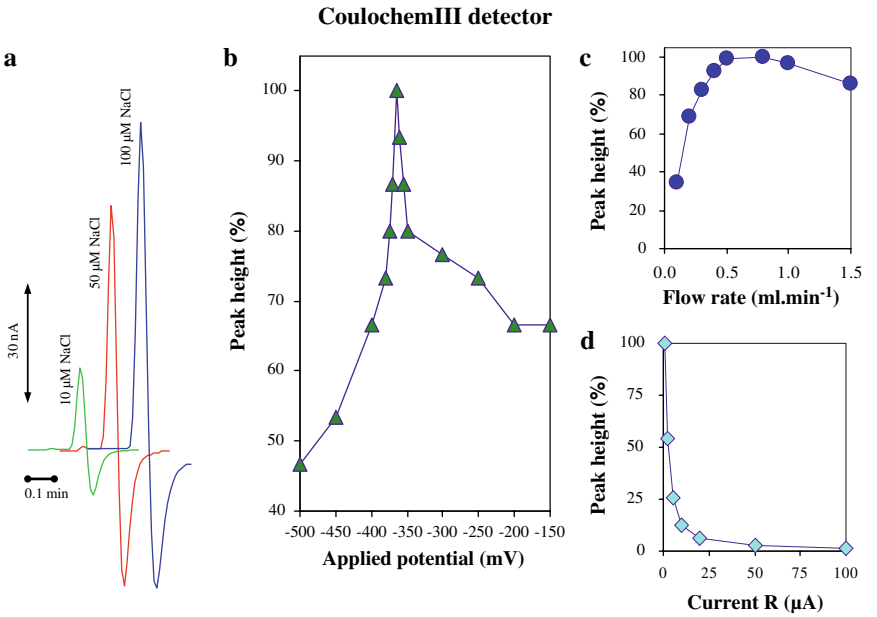

Fig. 3. The CoulochemIII detector. **a** FIA-ED output for 10, 50, and 100 µM chloride. **b** Hydrodynamic voltammogram obtained from chloride anions. Effect of **c** mobile phase flow rate and **d** "Current *R*" on response to chloride

Because mobile phase flow rate and other chromatographic conditions also have a marked effect on the signal, the effect of the flow rate of acetate buffer (pH 5.0) on the electrochemical signal of the chloride anions was studied (Fig. 2c). The results obtained showed that the best response was obtained at 1 mL min^{-1}. A calibration plot using cumulative response (calculated according to Petrlova et al. [47] and Potesil et al. [42]) was obtained under the optimized experimental conditions (working electrode potential −250 mV and mobile phase flow rate 1 mL min^{-1}). The detection limit (3 × S/N) for chloride ions was estimated to be 100 nM.

Detection of Chloride Ions using CoulochemIII Detector

The CoulArray requires relatively sophisticated instrumentation which cannot be miniaturized. In contrast with the Corona and CoulArray detectors, there are many technical possibilities of miniaturization of the amperometric CoulochemIII detector. For example, carbon electrodes can be miniaturized easily by use of thick-film technology [49, 50]. We tested the CoulochemIII for detection of chloride anions. The ions (100 µM) gave very repeatable peaks in the presence of 0.2 M phosphate buffer, pH 7.0 (for $n = 10$, the relative standard deviation, RSD, of signal height was 3%). The peaks obtained from 10, 50, and 100 µM NaCl are shown in Fig. 3a. To reduce the detection limit the experimental conditions were optimized. When the dependence of peak height on applied potential was measured a marked increase in the peak height was observed at a potential of −350 mV, with a maximum at −365 mV. The chloride signal then decreased (Fig. 3b). The dependence of chloride peak height on mobile phase flow rate was investigated at a potential of −365 mV. The signal increased gradually up to a flow rate of 0.5 mL min^{-1}, where the maximum signal was obtained, then decreased slightly (Fig. 3c). We then optimized the "Current *R*", one of the settings of the instrument [51]. "Current *R*" markedly affected the electrochemical signal of chloride ions (Fig. 3d). The highest responses were obtained at the lowest applied "Current *R*", but at the expense of increased noise, which negatively affected the analytical signal. We observed that the noise increased markedly when "Current *R*" was below 5 µA, so this value was used in subsequent experiments.

Mobile phase pH also affected the electrochemical response to chlorides, the highest peaks being obtained at pH 6 (Fig. 4a).

Under the experimental conditions working electrode potential −365 mV, "Current *R*" 5 µA, mobile phase 0.2 M phosphate buffer, pH 6, flow rate 0.5 mL min^{-1}, the dependence of chloride anion peak height on concentration was investigated (Fig. 4b). The signal was proportional to chloride anion concentration. Strictly linear dependence ($y = 53.473x + 0.0255$; $R^2 = 0.9976$) was observed in the range 0–100 nM (inset in Fig. 4b). The detection limit (3 × S/N) for chloride ions was estimated as 1 nM; the limit of quantification was estimated as 3 nM and the

relative standard deviation was below 5%.

In addition to NaCl as a source of chloride ions, $SrCl_2$, NH_4Cl, and CsCl were also tested. The effect of the different cations was investigated in two concentration ranges (75–800 nM and 5–60 μM). The results obtained are summarized in Table 1. The concentration dependences obtained were strictly linear. To examine the effect of cations on chloride signal the slopes (a) of the regression equations ($y = ax + b$) were compared to the slope measured for NaCl. The results obtained showed that strontium(II) ions enhanced the response of chloride ions by approximately 40% compared with NaCl. The effect of ammonium ions was strongly dependent on their concentration. In the lower concentration range the cations enhanced the signals but higher concentrations of these cations reduced the response to chloride (Table 1). The effect of caesium ions was similar to that of ammonium ions, i.e. higher concentrations of this cation reduced the peak height of chloride ions, and vice versa.

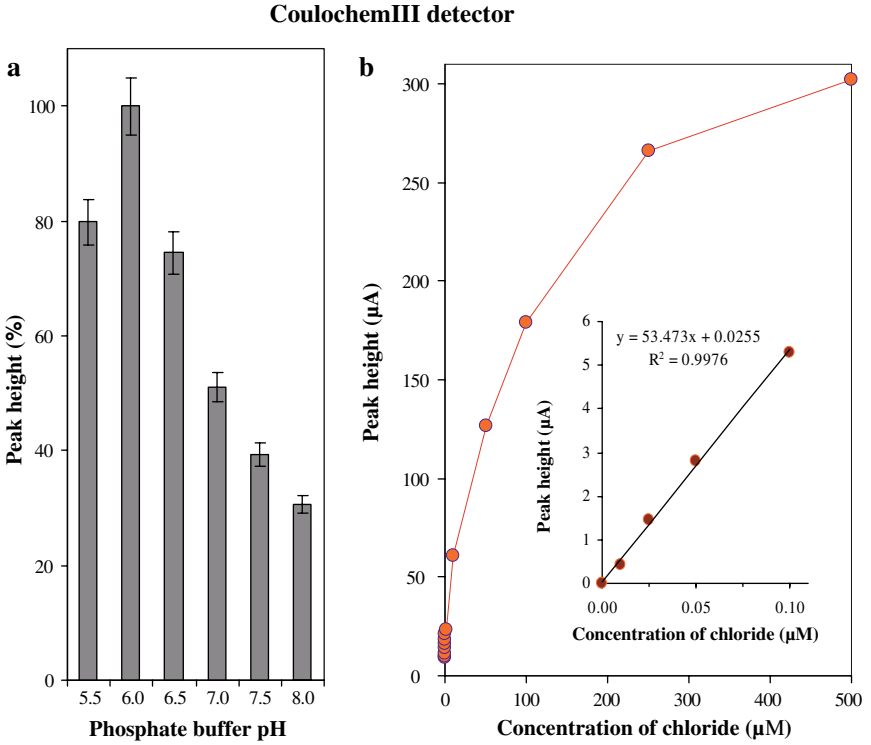

Fig. 4. The CoulochemIII detector. Dependence of chloride ion peak height on **a** mobile phase pH and **b** concentration of the ions. Inset: calibration plot in the concentration range 0 to 100 nM NaCl

Use of Haloalkane Dehalogenase for Detection of Halogenated Molecules

Many species of chemotrophic bacteria contain enzymes which are able to release halide ions from halogenated hydrocarbon molecules. Haloalkane dehalogenase (E.C. 3.8.1.5.) can be used to hydrolytically cleave the carbon–halogen bond, forming the corresponding alcohol, halide anion, and a proton [52]. These enzymes can be used for detection of chlorinated hydrocarbon pesticides. Chloride ions released by the enzyme can be detected by use of an electrochemical detector, as shown above. It is known that enzymatic activity can be affected by many factors, for example buffer composition, pH, ionic strength, and temperature. The haloalkane dehalogenase LinB from bacterium *Sphingobium japonicum* UT26 has been used to detect halogenated hydrocarbons [27]. The experimental conditions described by Nagata et al. [27] for the highest activity of this enzyme had to be modified in our experiments, because the presence of glycine buffer (pH 8.6), used for measurement of the kinetic properties of LinB, is not suitable for electrochemical analysis, because of the high background signal and the reduced sensitivity of the electrochemical response. The glycine buffer was therefore exchanged for borate buffer, pH 7.6. Under these experimental conditions, enzymatic release of chloride from 1-chlorohexane (1 mM) was investigated. The time course of the reaction was studied at room temperature. Under these conditions the chloride signal increased quickly up to 30 min, then increased moderately. The activity of the enzyme increased with increasing reaction temperature until a maximum was observed at 39 °C (Fig. 5a). The results obtained were in good agreement with data obtained by colorimetric detection of the activity of

Table 1. Effect of different cations on the peak height of chloride ions detected by use of the CoulochemIII detector

Salt	Regression equation; R^2		Effect of the cation (%)[a]	
	5–60 (μM)	75–800 (nM)	5–60 (μM)	75–800 (nM)
NaCl	$y = 26.543x + 4.3329$; 0.9995	$y = 4.6612x + 64.799$; 0.9990	100	100
$SrCl_2$	$y = 32.922x + 3.645$; 0.9993	$y = 7.3316x + 87.94$; 0.9969	124	157
NH_4Cl	$y = 22.281x + 3.9692$; 0.9992	$y = 5.7506x + 51.997$; 0.9996	84	123
CsCl	$y = 13.598x + 2.2281$; 0.9993	$y = 4.9022x + 59.553$; 0.9981	51	105

[a] NaCl peak height corresponds to 100%, the effect of the cation was estimated as the ratio (slope of the calibration plot for $SrCl_2$, NH_4Cl, or CsCl)/(slope of the calibration plot for NaCl) × 100

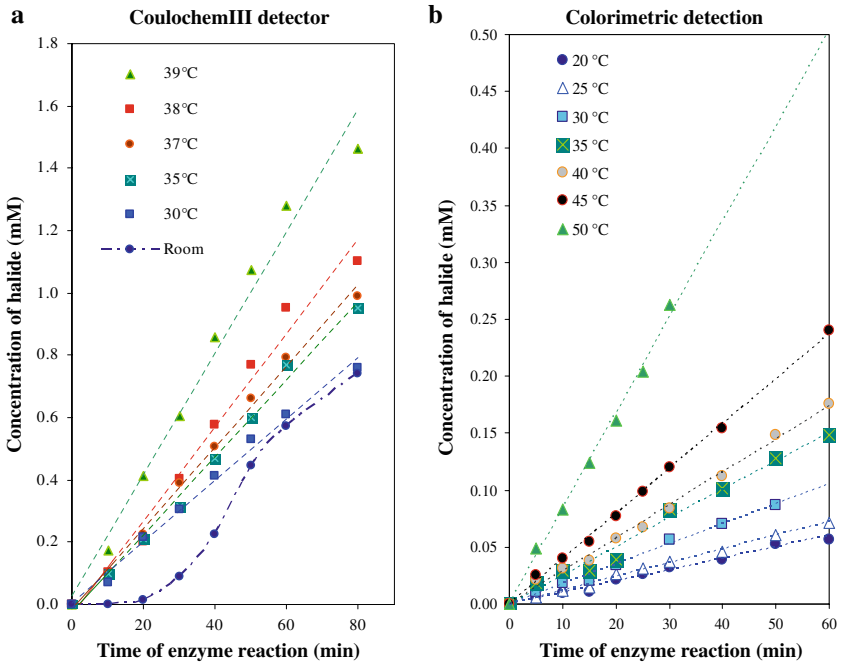

Fig. 5. **a** Signals from halide ions released by the enzyme LinB during enzymatic reaction with 1-halidehexane. The reaction was performed in borate buffer, pH 7.55, at 25, 30, 35, 37, 38, and 39 °C. **b** Measurement of DbjA dehalogenase activity at different temperatures. The concentration of halide ions released from the substrate 1-halidehexane was measured by the Iwasaki colorimetric method [36]

dehalogenation enzyme DbjA [53] from the bacterium *Bradyrhizobium japonicum* USDA 110 (Fig. 5b).

Conclusions

Many instruments have been proposed and developed for use in the analysis of organic pesticides. Here we report a new and unique means of analysis of these toxic compounds. Under optimized conditions we used enzyme-catalysed cleavage of a chlorinated hydrocarbon and sensitive electrochemical method to detect 1-chlorohexane. This procedure can be further tested for analysis of chlorinated hydrocarbons, including pesticides.

Acknowledgments

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