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High-performance liquid chromatography–ToxPrint: chromatographic analysis with a novel (geno)toxicity detection

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Abstract

In order to aid the monitoring of the overall quality of (surface) waters a new analytical approach has been developed, combining on-line solid-phase extraction, HPLC separation and effect-related detection. Compounds present in surface water or wastewater samples are extracted on-line with Oasis [poly(divinylbenzene–co-*N*-vinylpyrrolidone)] material and directly fractionated by reversed-phase HPLC. The eluent of the total chromatogram is collected on a microtitre plate in fractions of 1 min each. After evaporation and re-dissolvation in a suitable solvent, the (geno)toxicity of the individual fractions before and after enzymatic activation with S9, is determined with the umu test. In this way, harmful compounds can be detected and localized in the HPLC–diode array detection trace even without their identity and exact concentration being known at that moment. The method was developed using two test compounds, 4-nitroquinoline-*N*-oxide and 2-aminoanthracene. Compounds with mutagenic properties comparable to those of the test compounds can be detected from 0.1 μg/l, which is a concentration relevant for surface waters. The new analytical approach was successfully applied to various types of model samples, as well as real wastewater. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Water analysis; Detection, LC; Genotoxicity; Umu test; Environmental analysis; Aminoanthracene; Nitroquinoline-*N*-oxide

1. Introduction

In countries where surface water is one of the main sources in drinking water production, assessment and maintenance of the quality of surface water is very important. The quality of surface waters can be influenced by many factors, such as agricultural and industrial effluents, therefore the reduction and control of the emission of pollutants is of great importance. Target compound analysis with modern analytical techniques such as gas and liquid chromatography combined with different types of detection is often performed in order to detect and quantify known pollutants in both surface water and different types of wastewater. In order to ensure the quality of surface waters, extensive monitoring programs need to be carried out, in which hundreds of specific substances are tested for. For known pollutants, substance-specific risk assessment (with respect to toxicity or genotoxicity) can be achieved, based on the properties of the individual compounds. However, in addition to known pollutants, unknown

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contaminants may be present in water, thus leading to discrepancies between the results of the monitoring programs and the actual surface water quality. Identification of all compounds present in water is an impossible task, especially because water often contains natural compounds, which have little or no consequences for the drinking water production. In order to focus the identification effort to health and environmentally relevant substances, the hyphenation of chemical separation with effect related techniques, such as toxicity testing, is desired.

There are several examples in the literature, describing the determination of acute toxicity in fractions obtained by extraction [1,2] or high-performance liquid chromatography (HPLC) fractionation [3,4]. The toxicity is determined by measuring the decrease in luminescence of the *Vibrio fischeri* bacteria and has a relatively high detection limit, requiring the presence of toxic compounds almost at the mg/l level. Although this approach proved very suitable for assessing wastewater quality, for surface water and drinking water production in general, where compounds are more dilute, a different approach needs to be developed. In surface waters, dilute of (pro)mutagenic substances are often present. Such compounds may pose a health risk to humans and other living organisms upon long-term exposure, even at low concentration levels [5]. As one of the most important factors of influence on the surface water quality is wastewater, the addition of genotoxicity determination is being considered for the whole effluent environmental risk procedure in The Netherlands [6,7].

This paper describes a newly developed coupling of HPLC and genotoxicity determination with a sensitive umu test [7–9]. The compounds are extracted on-line with solid-phase extraction (SPE) and separated by HPLC. After the performance of the umu test in all fractions, the genotoxic compounds can be localised in the HPLC–UV trace. We named this method ToxPrint. Within the scope of this paper, the method and applications to various types of wastewater are described. The identification of the substances, responsible for the positive scores in some of the real-life samples presented here, and the determination of their exact concentration is out of the scope of this paper.

2. Experimental

2.1. General

The goal of this study was the development of a method for the localization of genotoxic compounds in the HPLC–UV traces of various water matrices. Therefore, in this stage, only the use of genotoxicty tests was considered. The test should be sensitive to a large spectrum of harmful substances present in low concentrations and should be rapidly and easily applicable. Two tests are available fulfilling these requirements: the Ames test [7] and the umu test [8,9]. For this application, the umu test was selected because its sensitivity is higher than the Ames test, it is faster and can be applied and measured on microtitre plates in 96 fractions at the same time [1,9]. The selection of the type of test determines the requirements for the extraction and HPLC fractionation. In this first stage an on-line SPE at pH 7 was selected as most suitable for this purpose. A reversed-phase HPLC fractionation is performed and a collection time of 1 min per fraction is considered as sufficient to resolve individual or a low number of compounds of similar polarity in each fraction.

In order to remove the HPLC effluent, the fractions collected on the microtitre plate are evaporated to dryness. Because of the different ratios of the organic solvent and water in each fraction, it is inevitable that during the evaporation step some fractions dry faster and earlier than others. In this way, loss of volatile compounds may occur. However, it is possible to minimize the volatilization by addition of a keeper solvent to each fraction.

The organic compounds have to be re-dissolved before the umu test is performed. The solvent has to be miscible with water, has to dissolve a large spectrum of organic compounds and should not introduce false mutagenicity during the test. For this application, a diluted solution of dimethyl sulfoxide (DMSO) in water is reported to yield the best results [9–11].

2.2. Chemicals

The HPLC-grade water is obtained from a Milli-Q system (Millipore, Etten Leur, The Netherlands). The

analytical column is flushed between two chromatographic separations with KH_2SO_4 (Baker, analyzed, Mallinckrodt Baker, Deventer, The Netherlands) in ultra-pure water in a concentration of 1 g/l. Acetonitrile is used as the organic modifier (Chromosolv for HPLC, gradient grade, Riedel-de Haën, Seelze, Germany).

Both the eluent and the KH_2SO_4 solution, used to rinse the system, are de-aerated using helium (Hoekloos, 99.999% pure) and placed under constant pressure (0.2 bar). The DMSO used is of GC quality (Merck, Darmstadt, Germany).

The test compounds are 2-aminoanthracene (2-AA) and 4-nitroquinoline-*N*-oxide (4-NQO) (Sigma–Aldrich, Zwijndrecht, The Netherlands).

2.3. Equipment

The samples are filtered over 0.2-μm regenerated cellulose material RC 58 (Schleicher & Schuell, Dassel, Germany). The HPLC–UV system consists of a Gilson 232-401 autosampler (Meyvis, Bergen op Zoom, The Netherlands), a gradient HPLC pump Model 250 (Perkin-Elmer, Gouda, The Netherlands) and a Model LC-95 UV detector (Perkin-Elmer) or a photodiode array detection (DAD) system Model 991 (Waters, Etten-Leur, The Netherlands).

The pre-concentration of the sample is carried out on a 20×3 mm I.D. column, packed with Oasis material (Waters). Oasis is a porous co-polymer [poly(divinylbenzene–co-*N*-vinylpyrrolidone)] with adsorption capacity for both lipophilic and hydrophilic compounds, HLB, 25–35 μm, 73–89 Å pores, 800 m^2/g. Experiments have shown [12] that this material can extract compounds with a broad range of polarities. Extraction recoveries of 75–125% can be assumed for most compounds eluting in the investigated range (11–45 min) of the chromatograms.

The pre-concentration column is mounted on the injection valve of the autosampler, instead of the sample loop. The sample is pumped over the pre-concentration column with a HPLC pump (Kipp Analytica, Delft, The Netherlands).

The analytical column is a 250×4 mm I.D. Inertsil ODS-2, 5 μm material from GL Sciences (Chrompack, Middelburg, The Netherlands), the guard column is 10×2 mm I.D. packed with pellicular C_{18} material, 25–35 μm (Chrompack). The analytical column and the guard column are maintained at a temperature of 8°C in a column thermostat (W.O. Electronics, Applied Science Group, Emmen, The Netherlands).

A Model 202 fraction collector is fitted at the outlet tubing of the UV detector (Meyvis). The fractions are collected in a polyethylene 96-well plate, with 1.2 ml/well capacity (Aurora Borealis Control, Schoonebeek, The Netherlands).

2.4. Analytical method

The analytical method is based on the reversed-phase HPLC separation of herbicides and pesticides [13] and a HPLC–DAD fingerprint method [14]. The sample is pre-concentrated on-line with a HPLC pump, the sample size is related to the water quality and varies from 20 to 100 ml. The on-line pre-concentration, HPLC separation and fractionation are automated using a column-switching system (see Fig. 1). A linear gradient of acetonitrile and water is used, for details see Table 1. Experiments have shown that the best separation results are obtained if the analytical column is left to equilibrate for 20 min after each analysis (last step of the gradient). The compounds separated by HPLC, are detected by DAD and in the applications described here, fractions 11–46 are collected on a 96-well plate using a fraction collector. The fraction collection time is 1 min/well (e.g., compounds eluting between 10 and 11 min are collected in fraction number 11). DMSO is added as a keeper solvent and the HPLC eluent is evaporated under a gentle stream of nitrogen. The complete fractionation procedure is performed two times for each sample.

In one of the fraction sets, the umu test as such is performed, the duplicate fractionation is used to perform the test with the addition of S9 liver homogenate. The enzymes in the liver of higher organisms can disarm harmful substances (metabolic deactivation). Alternatively, non-mutagenic substances may break down and become genotoxic by this enzyme (metabolic activation).

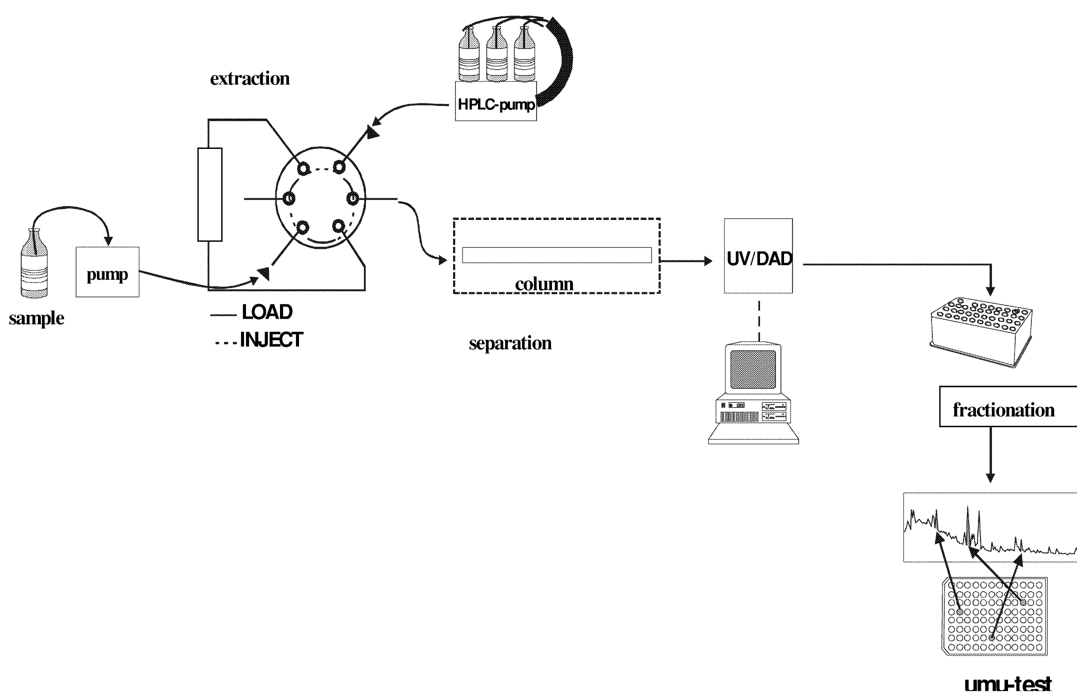

Fig. 1. Overview of the ToxPrint method: on-line pre-concentration, separation, detection, fractionation and umu test.

2.5. Umu test

The umu test is performed according to the German standard methods for the examination of water, wastewater and sludge [13]. The test utilises a sensitive mechanism of bacteria to detect DNA damage. As a reaction on the detected genetic disruption, bacteria (*Salmonella typhimurium*, TA1535/pSK1002) activate a complex enzymatic system, the SOS, to repair the damage. This test measures the expression of SOS umu-C genes induced by genotoxic agents. The engineered bacterial strain used has the gene for the enzyme β-galactosidase behind the SOS promoter, instead of the SOS genes. In this way induction of the SOS repair system by genotoxic agents can be measured by a photometric determination of the β-Gal enzyme activity. In addition to the umu test performed in the fractions, the quality parameters of the test are also determined simultaneously: the response of the test is determined in (I) a dilute DMSO solution in water with bacteria (blank), (II) water with only bacteria added, (III) DMSO solution with only the medium added and (IV) DMSO solution with the medium, bacteria and the performance control compounds 2-AA and 4-NQO, added in four different concentrations. In addition to quality control purposes, a dose–effect correlation (see also Results and discussion) is calculated for the performance control compounds. The average of the response of I–III is used as the blank correction for all experiments in the same series. Quantification of the observed effect is

Table 1
Gradient used for the elution of samples in the HPLC–ToxPrint approach

Time (min)	Flow (ml/min)	A (%)	B (%)
Separation			
0	0.7	90	10
40	0.7	20	80
42	0.7	0	100
Conditioning			
52	1.0	0	100
54	1.0	90	10
75	1.0	90	10

A=Ultrapure water, B=acetonitrile.

therefore relative, however, it can be used to access the effect of the various fractions in the chromatograms. The results of the test are available within 2–3 h. In this first stage of the research, the umu test was performed manually. At present, an automated version of the method is being developed.

2.6. Interpretation of the results

The application of HPLC–ToxPrint results in a two-dimensional figure with the retention time (fraction number) on the x-axis, and the response of the bacteria (after blank correction), expressed as the UV absorbance in mV, on the y-axis. The interpretation of the results is always based on comparing the signal of the individual fractions in one sample with the trends observed for that particular sample: positive or negative ‘‘peaks’’, see Fig. 2.

Four different results can be distinguished: (I) the trend in bacterial growth and genotoxicity is not disturbed and is the same as in the blank, the fraction shows no effect; (II) a clear minimum (a decrease by more than 0.1 on the presented scale) in both the bacterial growth inhibition and in the umu test results indicates a toxic effect of the fraction (toxic to the used bacteria, therefore the observed mutagenicity is lower than that of the other fractions or the blank); (III) a visible maximum (increase) of the genotoxicity signal, which is in some cases accompanied by a decrease in the bacterial growth, indicates a genotoxic fraction; and (IV) if only inhibition of bacterial growth is observed, this fraction is toxic, but the results concerning the mutagenicity are inconclusive.

3. Results and discussion

3.1. Positive control compounds and quantification

The aim of the developed method is the survey for occurrence and localization of mutagenic compounds in HPLC chromatograms. 4-NQO and 2-AA were selected as the test compounds for the performance of the umu test and the developed method. These compounds [calculated log K_{ow} (K_{ow}=octanol–water partition coefficient) 0.82 and 3.43, respectively] were diluted in four different concentrations on the microtitre plate with and without the addition of S9 enzyme. The performance of the test resulted in a dose–effect correlation, which can be expressed as a calibration curve (Fig. 3). 4-NQO is a strongly mutagenic compound and a standard solution gave a clear response in both mutagenicity and inhibition of bacterial growth without the addition of S9. In the presence of S9 enzymes, the response in mutagenicity decreased by a factor of two with respect to the results without S9. With S9, there was no inhibition of bacterial growth. 2-AA is a (pro)mutagenic compound and showed no mutagenicity and no growth inhibition without the S9 extract. However, if these

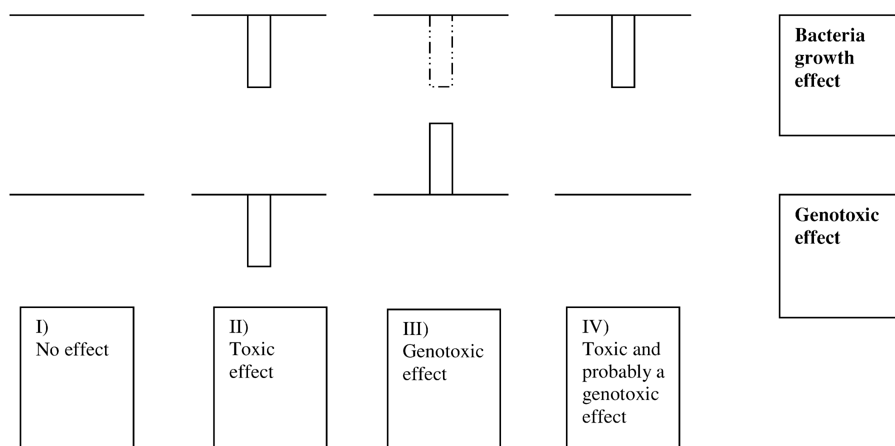

Fig. 2. Interpretation of the umu test results. Bar bellow the horizontal line: decrease, bar above the horizontal line: increase in the response; if compared to the blank. For more details see Experimental.

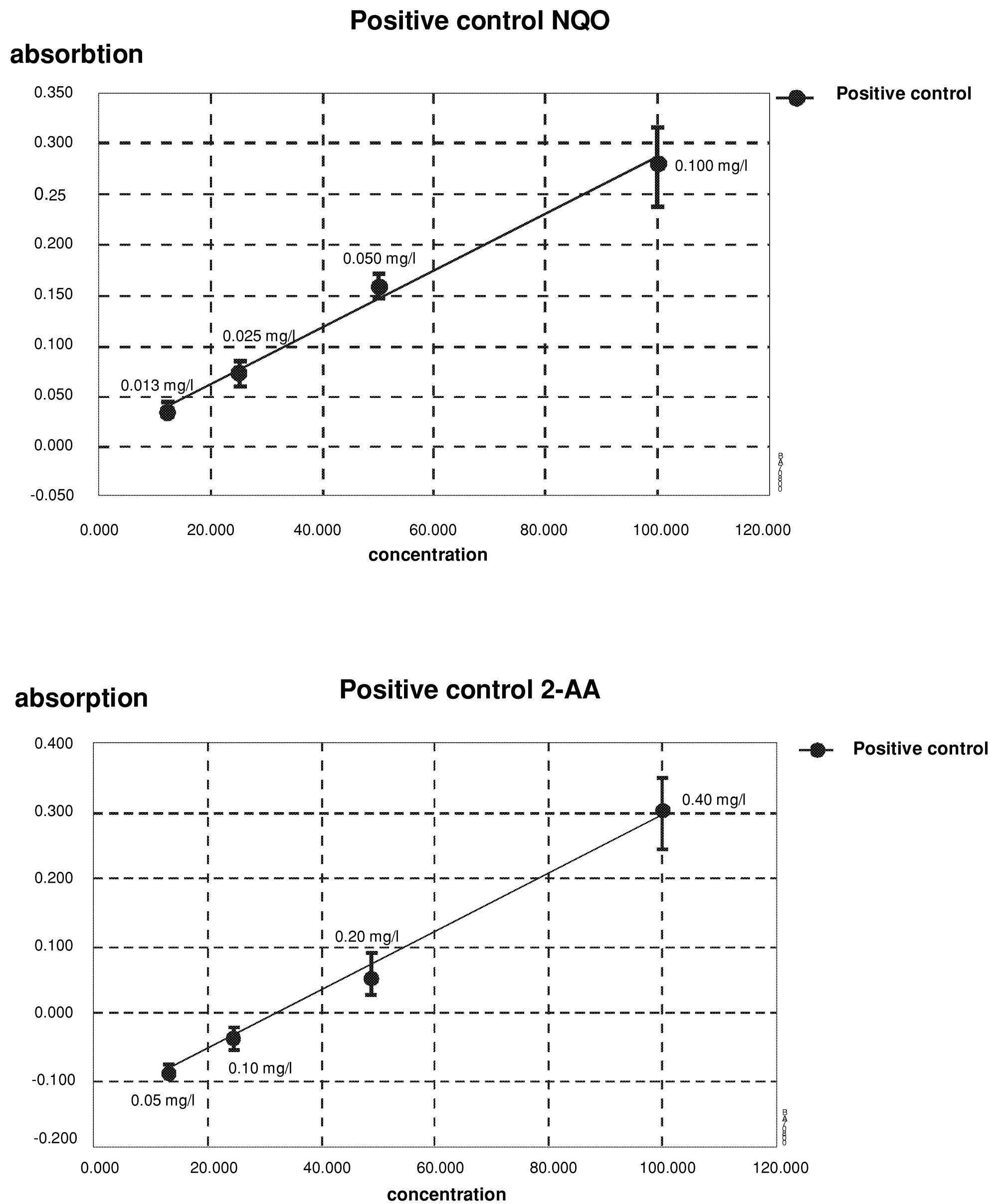

Fig. 3. Dose–effect correlation using two model compounds, 4-NQO (without S9) and 2-AA (with S9). On the *x*-axis the concentration of the model compound in the well is depicted, the *y*-axis represents the absorbance (in mV).

enzymes were added, 2-AA was activated and showed a strong response in terms of genotoxicity.

Based on the effect–dose correlation, sample volume and the concentration factor, the estimated limit of detection in the water sample was calculated for 4-NQO (0.03 μg/l) and 2-AA (0.1 μg/l). This means that depending on their properties, (pro)mutagenic compounds present in water samples can be detected at a concentration level of approximately 0.1 μg/l. The selected compounds have a very strong effect therefore the expected level of detection would be in the low μg/l range. Even though the

method was developed strictly for indication purposes, extra valuable information, estimation of the concentration of the (unknown) detected harmful compounds can be performed, assuming the same recovery and comparable genotoxic properties for the unknown compounds, as those of the used positive controls.

In addition to fortified surface and drinking water samples, used to test the performance of the developed approach, the method was also applied to several wastewater samples. It should be noted that the dissolved organic carbon (DOC) content of this type of samples is much higher than the DOC of surface waters, leading to possible overloading of the analytical column, and therefore lower resolution in the chromatograms.

3.2. Fortified surface water

Surface water was fortified with 1.8 μg/l of NQO and 5.6 μg/l 2-AA and analyzed with the developed method. The results in Figs. 4 and 5 show the bacterial growth related to the different fractions (Fig. 4A and Fig. 5A), the genotoxic effect (Fig. 4B and Fig. 5B) and the corresponding chromatograms at 230 nm (Fig. 4C and Fig. 5C). In the experiments without S9 enzymes, the fraction (No. 28) containing 4-NQO shows a clear bacterial growth inhibition (toxic effect, Fig. 4A), as well as mutagenicity response (Fig. 4B). For the 2-AA fraction (No. 46), no response in either of the tests has been observed. This is in agreement with the effects observed for the positive controls corresponding with this fortification level. In the experiments with S9, no significant response was observed in inhibition of the bacterial growth (Fig. 5A), both test compounds show a clear signal in the mutagenicity test (Fig. 5B). In addition to a slight matrix effect on the performance of the test, 2-AA partially decomposed under the influence of light, and gave a lower response than was expected based on the corresponding positive control. The response of 4-NQO in the experiments with S9 increased instead of decreasing, indicating a coelution of a pro-mutagenic substance together with the test compound, invisible to UV, which was activated by the S9. The chromatograms, Fig. 4C and Fig. 5C show that large sample volumes such as 100 ml can result in overloading the analytical column with humic acid and other matrix compounds. Experiments with fortified drinking water (not shown) gave very similar results.

3.3. Municipal wastewater

Municipal wastewater without addition of the test substances was analyzed. In the experiments without S9 (Fig. 6A), no distinctive response in the bacterial growth inhibition was observed. In fraction 19 (corresponding to a retention time of approximately 19 min), an isolated genotoxic response was observed (Fig. 6B). With S9, inhibition of growth was observed in fractions 19 and 20 (Fig. 6C) and the mutagenicity response of fraction 19 was even higher than in the experiments without S9 (Fig. 6D). In the chromatograms of this sample, the UV absorbance of the matrix was very high, no distinctive peak could be observed with a retention time between 18 and 19 min (Fig. 6E).

In order to determine the reproducibility of this method, the experiments were repeated and only fractions 18–20 were collected. As shown in Fig. 7, the results were reproducible. Without S9 a clear mutagenic response can be observed in fraction 19, this response approximately doubles in the experiment with S9. This example shows the power and the complementary value of effect-dependent detection, next to conventional (UV, DAD) detection techniques in HPLC. In addition to the determination of, in this case, mutagenicity of known and unknown UV-absorbing compounds, harmful compounds, invisible to UV, can be detected in HPLC chromatograms. This is in agreement with the results described in the literature [3], where toxic compounds, not detected by conventional detection techniques, were detected by the means of toxicity tests.

3.4. Industrial wastewater

An industrial wastewater sample was analyzed by the developed technique. Because of the very high DOC content of this particular sample, only 20 ml was extracted in order to avoid the overloading of the analytical column. The results of experiments without S9 are shown in Fig. 8A and B. The data for bacterial growth inhibition show a varying response throughout the chromatographic fractions. A signifi-

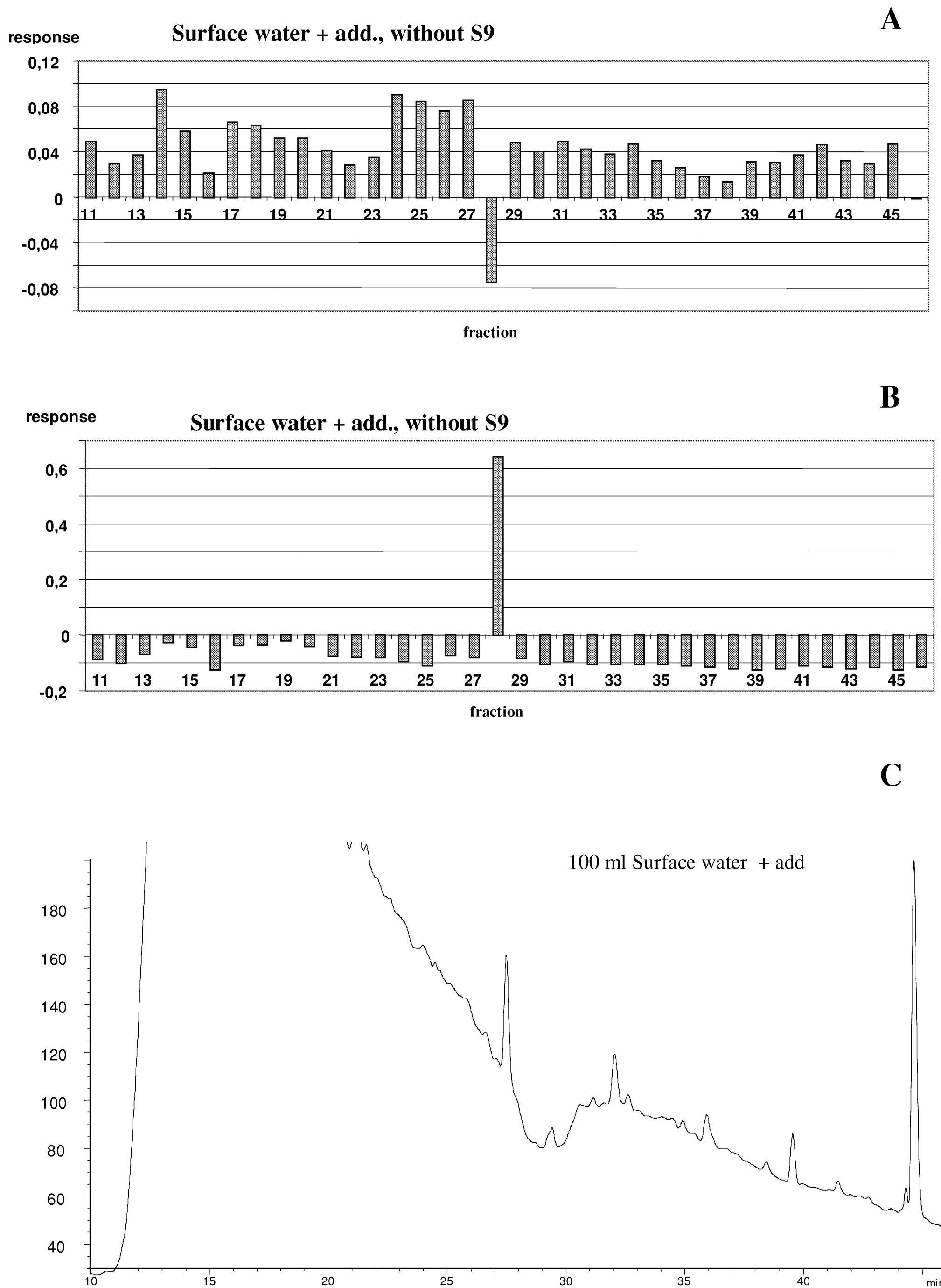

Fig. 4. Results of effect related testing in HPLC fractions of 100 ml of surface water fortified with 4-NQO and 2-AA, test results without S9 activation: growth inhibition (A), genotoxicity (B), HPLC–UV chromatogram at 230 nm (C). On the *x*-axis the fraction number (retention time) is shown, the *y*-axis represents the absorbance (in mV).

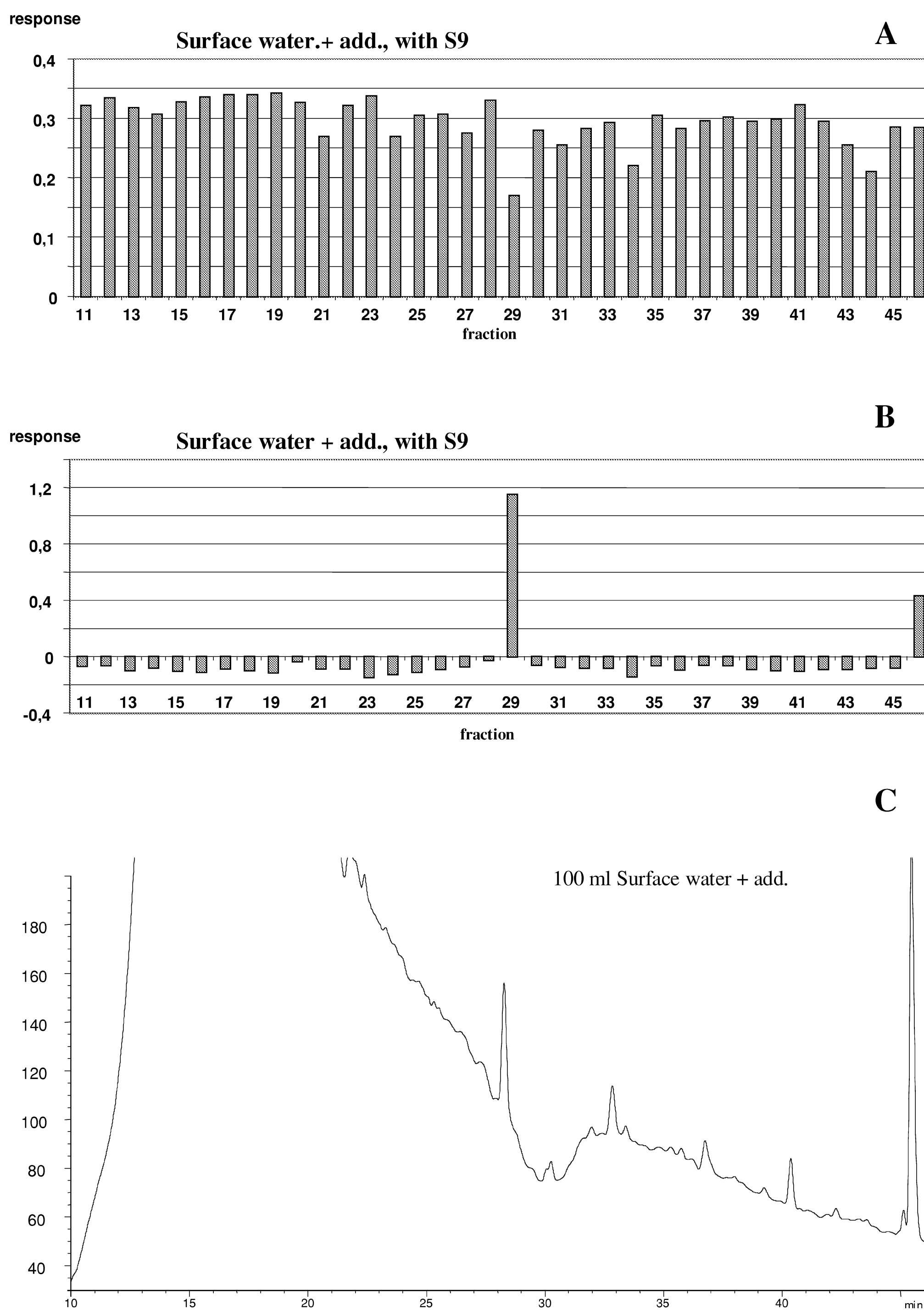

Fig. 5. Results of effect related testing in HPLC fractions of 100 ml of surface water fortified with 4-NQO and 2-AA, test results with S9 activation: growth inhibition (A), genotoxicity (B), HPLC–UV chromatogram at 230 nm (C). On the *x*-axis the fraction number (retention time) is shown, the *y*-axis represents the absorbance (in mV).

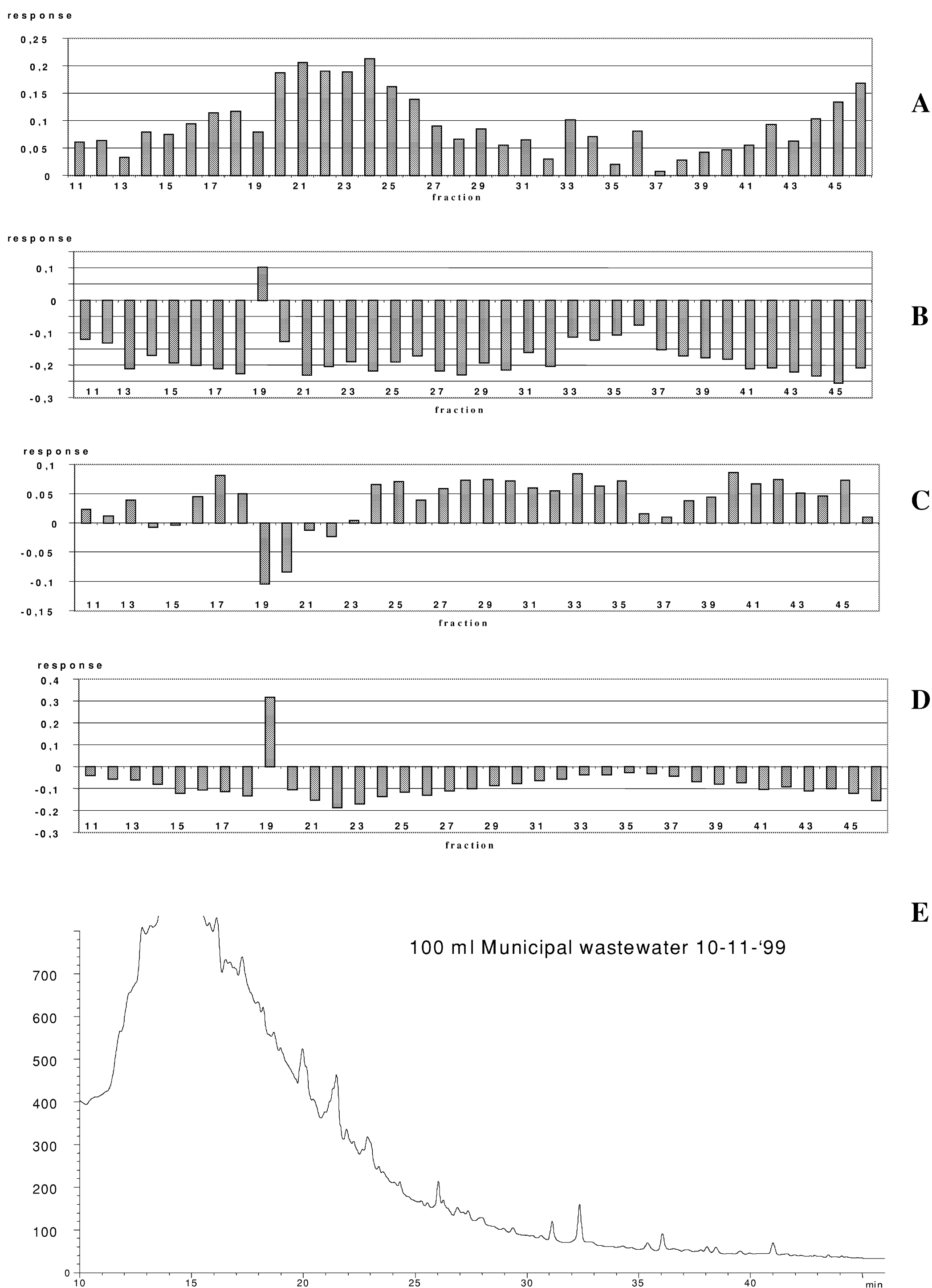

Fig. 6. Results of effect related testing in HPLC fractions of 100 ml municipal wastewater sample: growth inhibition without S9 (A), genotoxicity without S9 (B), growth inhibition with S9 (C), genotoxicity with S9 (D) and HPLC–UV chromatogram at 230 nm (E). On the *x*-axis the fraction number (retention time) is shown, the *y*-axis represents the absorbance (in mV).

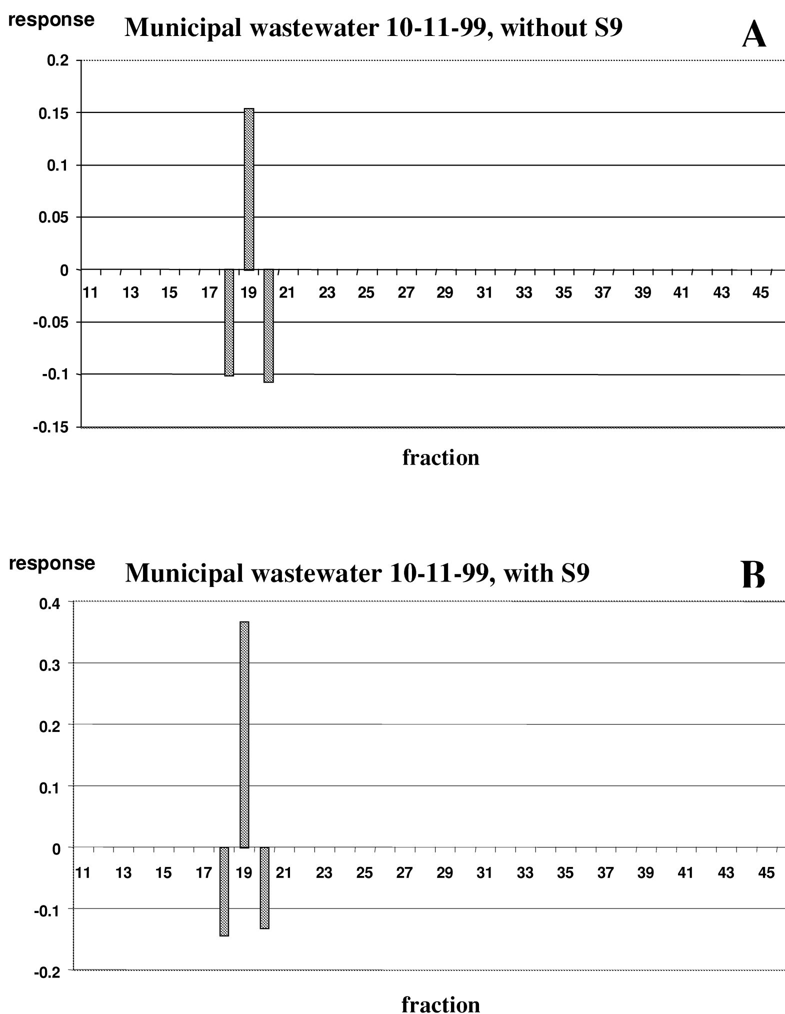

Fig. 7. Isolated genotoxic fractions of municipal wastewater. Results without (A) and with (B) metabolic activation with S9. This fractionation was performed 2 weeks after the experiment shown in Fig. 6.

cant decrease in the growth was visible in fractions 37 and 41. The mutagenicity test indicated the presence of harmful compounds in fractions 29, 34, 35, 41, 42, 43 and 44. At approximately 29 min a significant double peak was visible in the UV chromatogram (at 230 nm). Apparently, the first eluting compound had mutagenic properties (without S9). No distinct peaks were visible in the chromatogram corresponding to the mutagenic response in fractions 34 and 35 (Fig. 8E). The highest mutagenicity was determined in fractions 41 and 43. Based on the dose–effect correlation determined for 4-NQO and the concentration factor an estimate of the concentration of the compounds can be made, assuming similarity in the genotoxic effect. The absorbance of 0.35 and 0.30 in fractions 41 and 43 corresponded to the response of approximately 1.6 and 1.3 μg/l of 4-NQO in the wastewater sample. Based on the results, it cannot be concluded whether the (lower) response in the adjacent fractions 42 and 44 is caused by the splitting of the eluting compounds into two fractions or other compounds resolved in these fractions. In the UV chromatogram, no evident peaks were observed at the corresponding retention times. For this phenomenon, two explanations are plausible: UV absorbance of the corre-

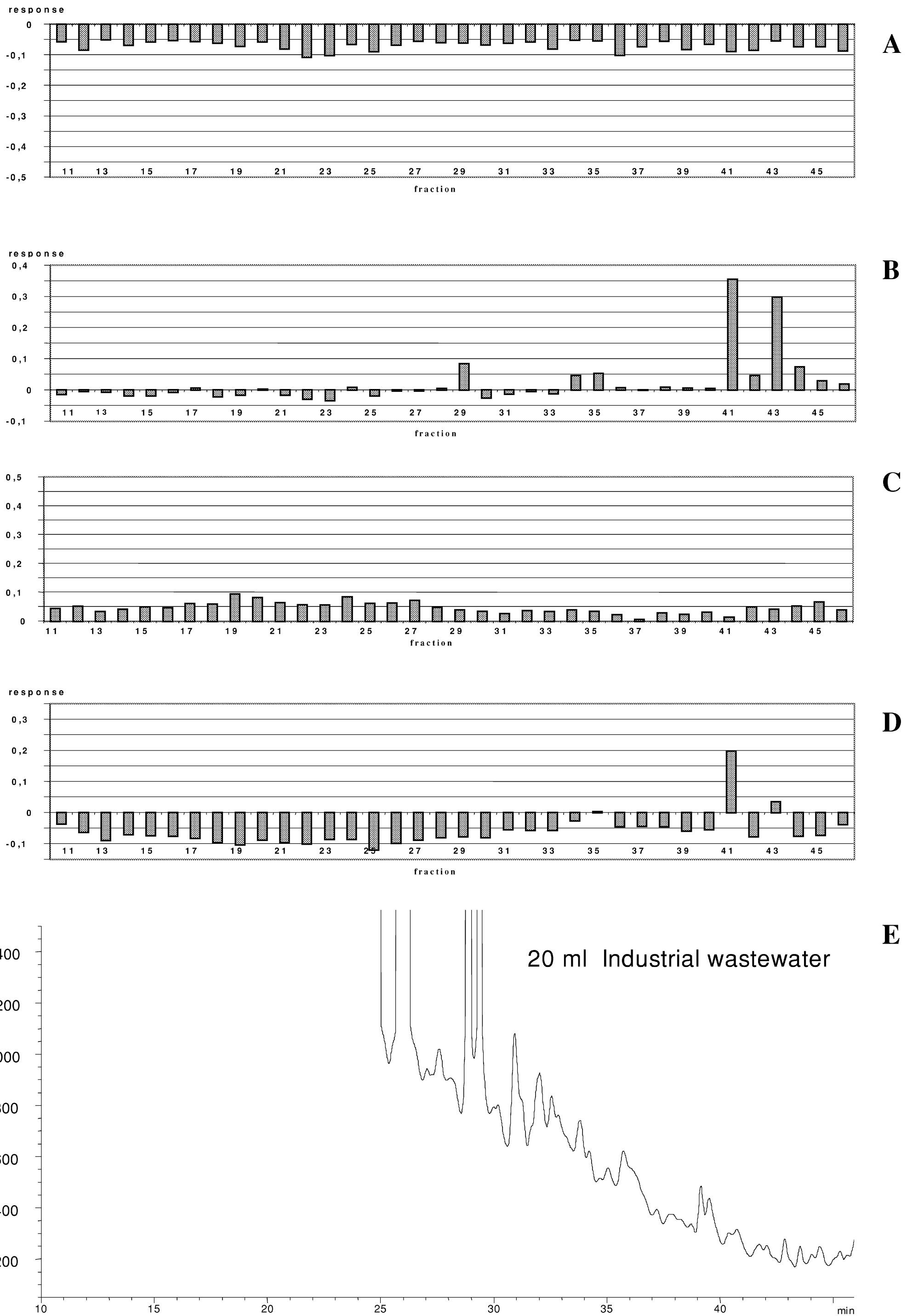

Fig. 8. Results of effect related testing in HPLC fractions of 20 ml of industrial wastewater sample: growth inhibition without S9 (A), genotoxicity without S9 (B), growth inhibition with S9 (C), genotoxicity with S9 (D) and HPLC–UV chromatogram at 230 nm (E). On the x-axis the fraction number (retention time) is shown, the y-axis represents the absorbance (in mV).

sponding compounds is lower than that of the test compounds or the mutagenic effect caused is very high, even at low concentrations. As shown in Fig. 8C, after activation by S9, no significant response was observed for the bacterial growth inhibition.

The mutagenicity of all fractions observed in the experiment without S9 strongly decreased (Fig. 8D). An evident response remained visible in fractions 41 and 43, but was much lower than before. This suggests a deactivation of the compounds mutagenic properties by the enzymes, as is the case with one of the test compounds, 4-NQO.

3.5. Hospital wastewater

Results of the analysis of hospital wastewater are shown in Fig. 9. Without S9, both the growth inhibition (Fig. 9A) and the mutagenicity response (Fig. 9B) showed that this sample had a different character than the analyzed municipal and industrial wastewater. Striking is, that larger part of the mutagenic and toxic response was located in the part of the chromatogram, which showed a decrease in the total UV response (Fig. 9E). Fractions 25 to 41 showed a visible toxic effect. Furthermore, a gradual increase in genotoxicity was observed from fraction 20, reached a maximum in fractions 32–35 and then slowly decreased again. Due to the obvious toxic effect of these fractions, the observed genotoxic effect is inconclusive, however the results implied that this sample contained numerous compounds with different polarities, not all UV absorbing, that have toxic and/or genotoxic properties. This is quite logical considering the variety of chemicals used in hospitals. With S9, based on the decrease in mutagenicity (Fig. 9D), we assumed that part of the compounds present in fractions 20–45 underwent metabolic deactivation. However, the lower response in the umu test could also have been influenced by the visible increase in toxicity (Fig. 9C) when S9 was applied.

It should be noted that the applications of the HPLC–ToxPrint described here were performed in order to test the performance of the developed method, before further automation and routine application. As far as the observed genotoxic fractions in the real samples are concerned, no further course of action was taken. Further identification of the mutagenic fraction(s) is out of the scope of this paper and will be discussed elsewhere.

4. Conclusions

A new powerful tool for the monitoring of the overall quality of surface water and wastewater effluents was developed, by successfully combining an on-line SPE–HPLC fractionation with a sensitive mutagenicity test. The response in the umu test and the bacterial growth can be used to assess both genotoxic and toxic properties of compounds, enabling the detection of (pro)mutagenic compounds in the low μg/l range.

Several examples show that effect-based detection can be used as a powerful complementary technique in combination with UV detection. Quite often, harmful compounds, invisible to UV detection due to lack of absorbance or a high matrix background, are detected by their mutagenic response. In order to broaden the application possibilities to determination of genotoxicity in drinking water production, further research, concerning automation of the procedure, alternative solvents, isolation and improvement of the overall sensitivity, is currently in progress. The approach described here, can further be combined with the identification of unknown (pro)mutagenic compounds with LC–MS–MS.

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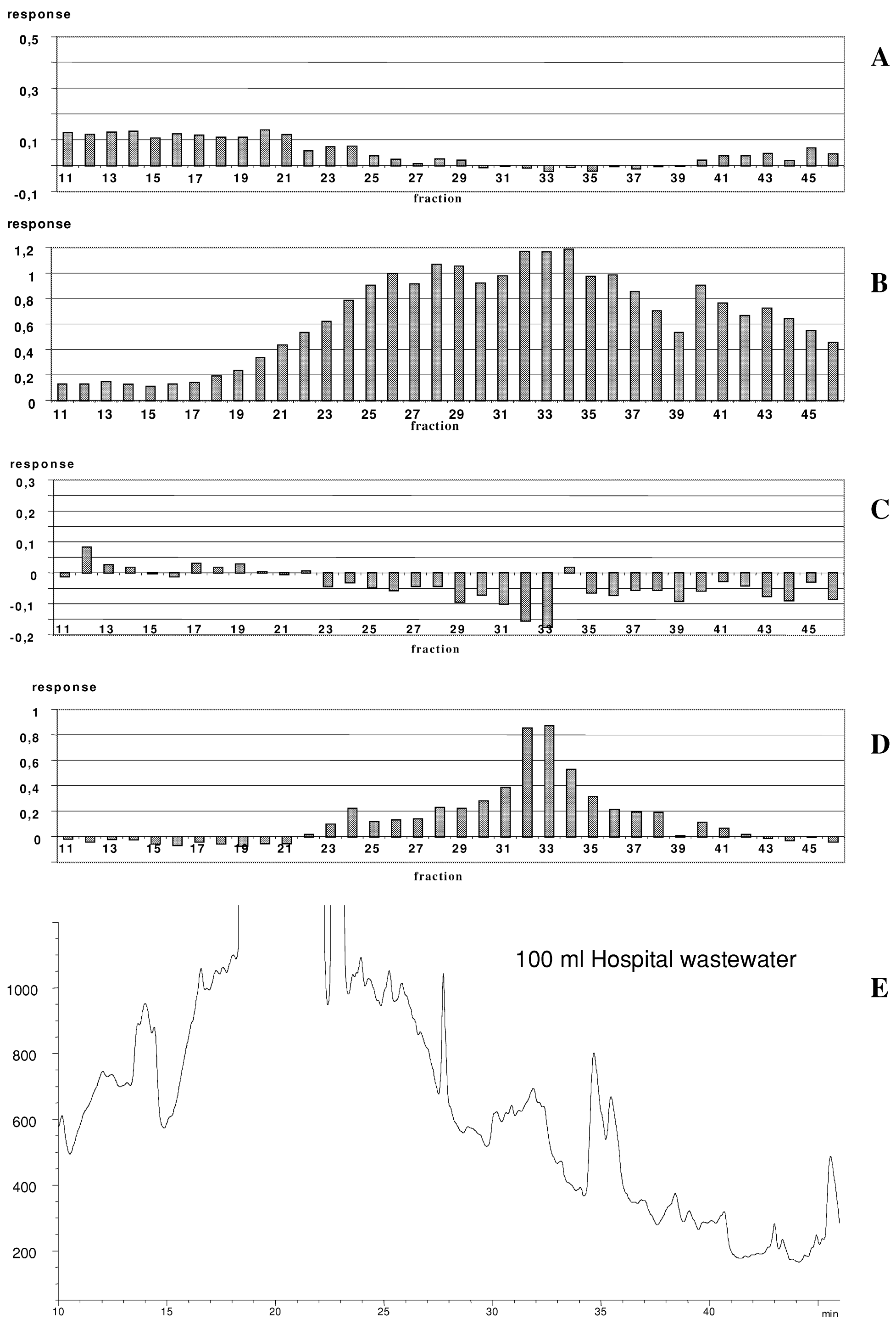

Fig. 9. Results of effect related testing in HPLC fractions of 100 ml hospital wastewater sample: growth inhibition without S9 (A), genotoxicity without S9 (B), growth inhibition with S9 (C), genotoxicity with S9 (D) and HPLC–UV chromatogram at 230 nm (E). On the *x*-axis the fraction number (retention time) is shown, the *y*-axis represents the absorbance (in mV).

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