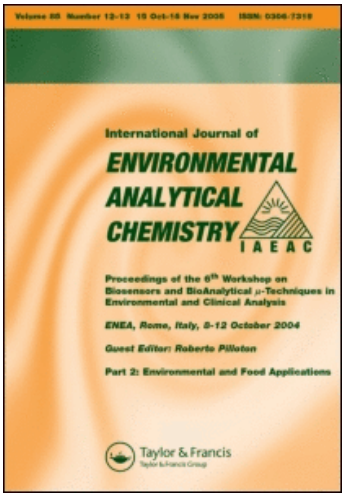

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Development of an HPLC-Method for the Determination of E-2-Nonenal in Beer using Column Switching Techniques†

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E-2-nonenal is an important compound formed during ageing of beer. It has a flavour threshold value of about 0.1 μg/l. A method is described for isolation of E-2-nonenal from beer by solid phase extraction. After extraction E-2-nonenal is derivatized with dansylhydrazine and analyzed with reversed phase HPLC using column switching techniques. The combination of on-line preconcentration of the derivative and heartcutting enables the analysis of E-2-nonenal in beer at natural levels.

KEY WORDS: E-2-nonenal, reversed phase HPLC, on-line preconcentration, dansylhydrazine, column switching.

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INTRODUCTION

Although during ageing of beer several compounds are formed, especially the compound E-2-nonenal (E-2-N) is responsible for the characteristic stale flavour of beer[1,2,3] This unsaturated aldehyde has a flavour threshold in beer in the range of 0.05–0.1 μg/l.[4] This implies that a method for the determination of E-2-N in beer must have a limit of determination in the mentioned range, in order to be valuable in research and quality control.

Due to the very low concentration of E-2-N in beer a considerable preconcentration combined with an improvement of its detectability e.g. by derivatization is unavoidable. In 1974 Wang and Siebert[3] published a method for the determination of E-2-N in beer, but the method was rather laborious. Strating *et al.*[5] developed a method for the isolation of E-2-N from beer based on liquid–liquid extraction which was combined with adsorption chromatography as pre-concentration. The residue of this pretreatment was subsequently analyzed by means of two-dimensional gaschromatography. Although the sensitivity was adequate, the method was still rather laborious. Greenhoff and Wheeler[6] published an HPLC method for the analysis of aldehydes in beer including E-2-N. After isolation by vacuum distillation the aldehydes were derivatized with 2,4-dinitrophenylhydrazine, concentrated and separated by reversed phase gradient chromatography. Recently Piendl *et al.* reviewed the different methods for the analysis of carbonyl derivatives by HPLC.[7]

In this communication we describe the preliminary results of a method in which E-2-N is isolated from beer by solid phase extraction followed by pre-column derivatization with dansyl-hydrazine.[8]

The resulting nonenal derivative is analyzed by reversed phase HPLC using column switching techniques and fluorescence detection.

EXPERIMENTAL

Apparatus

The chromatographic system consisting of two Kratos 400 HPLC

pumps, two Milton Roy minipumps, two column switching units (Must, Spark, The Netherlands), each with two six port valves (Rheodyne model 7010) and a Kratos FS 970 fluorescence detector is depicted in Figure 1. Valve switching was microprocessor controlled using a Kipp & Zonen (Delft, The Netherlands) model 5140 programmer. Excitation was at 250 nm and for emission a 470 nm cut off filter was used.

A modified $\frac{1}{16}''$ Swagelok union tee was used as a mixing device. The time schedule for the position of the different valves is depicted by means of drawn and dashed lines.

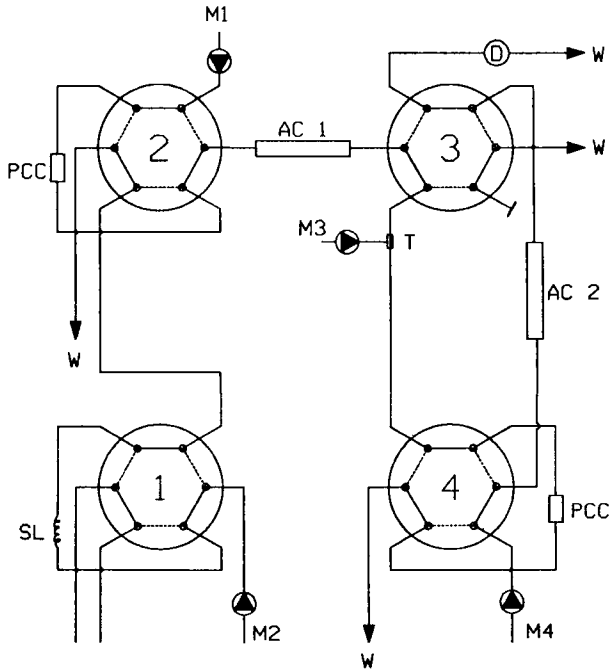

FIGURE 1 M = mobile phase, W = waste, D = detector, AC = analytical column, T = modified union, T, PCC = pre-concentration column, SL = sample loop.

Valve switching time schedule after loading injector (valve 1):

Time (sec)	Valve 1	Valve 2	Valve 3	Valve 4
0	——	---	---	——
720	——	——	---	——
1450	——	——	——	——
1580	——	——	——	---

Chemicals

Methanol, acetonitrile (HPLC grade), sodium acetate, acetic acid, potassium hydrogen phosphate and phosphoric acid (85%) were from E. Merck A.G., (Darmstadt, GFR). Dansylhydrazine was obtained from Pierce. E-2-nonenal was supplied by Ventron (Karlsruhe, GFR).

Stationary phases and columns

The precolumns used were 10×2 mm I.D. stainless steel cartridges (Chrompack, Middelburg, The Netherlands) hand-packed with 10 μm R Sil C_{18}D (Alltech-RSL, Gent, Belgium). A 25 cm $\times$ 4.6 mm LiChrosorb 10 RP 18 and a 12.5 cm $\times$ 4 mm LiChrosorb 5 RP 18 (Hibar) both from E. Merck were used as analytical column. SepPak C18 cartridges were from Waters Assoc. (Etten Leur, The Netherlands).

Chromatographic conditions

Mobile phases:

M1 = methanol/0.05 M sodium acetate, 80/20 (v/v)

M2 = methanol/0.05 M acetate buffer pH 4.0, 50/50 (v/v)

M3 = 0.05 M acetate buffer pH 4.0

M4 = acetonitrile/0.05 M acetate buffer pH 4.0, 70/30 (v/v)

The flow rate amounted to 1.0 ml/min.

Sample preparation

50 ml of cooled beer (5–10°C) was passed through a preconditioned SepPak C18 cartridge according to a method described elsewhere.[5] The cartridge was then washed with 10 ml of demineralized water and centrifuged for 5 min. at about 1000 g. Then the cartridge was connected upside down to a screwcap vial using a PTFE lined septum as sealing. By means of a glass syringe 1 ml of methanol was flushed through the cartridge and collected in the vial. The whole set-up (cartridge + vial) was centrifuged for 5 min. at 1000 g. After removal of the cartridge 100 μl of a dansylhydrazine solution

(4 mg/ml methanol) and 50 μl of glacial acetic acid were added, the vial was closed, the content mixed and stored in the dark at room temperature for at least 3 h. Then the reaction mixture was diluted with 1400 μl of a 0.05 M acetate buffer pH 4.0 and the mixture filtrated (0.5 μm). Finally the whole sample (about 2500 μl) was injected into the HPLC system.

RESULTS AND DISCUSSION

Sample preparation

The isolation of E-2-N from beer by means of solid phase extraction has advantages over solvent extraction. Formerly it was noticed[5] that it is rather difficult to evaporate the extraction solvent without any loss of the analyte (E-2-N). By using SepPak C18 cartridges E-2-N is easily isolated. During this step several short chain carbonyls, carbohydrates etc., which also react with dansylhydrazine, are removed because of their low retention on the C18 support material of the cartridge.

The water hold-up of the cartridge which can disturb the desorption step is efficiently removed by centrifuging the cartridge. By backflushing the cartridge with 1 ml methanol the adsorbed compounds are recovered and collected in a screwcap vial. Centrifuging of the cartridge connected to the vial increases the recovery of the methanol and thus of the analyte.

Derivatization

The E-2-N isolated from the beer is derivatized by reaction with dansylhydrazine. Optimal temperature and time for derivatization were determined by following the reaction in a real beer sample spiked with 17 ppb of E-2-N. By using this relatively high concentration of E-2-N it was possible to analyze 20 μl aliquots from the sample after different reaction times. This avoided the need for making a heartcut, because the peak was easily determined on the tailing front peak. Figure 2 gives the relative peak height of E-2-N as a function of the reaction time at different temperatures. It can be seen that reaction at room temperature results in a maximum peak height and a reaction product that is stable for several hours.

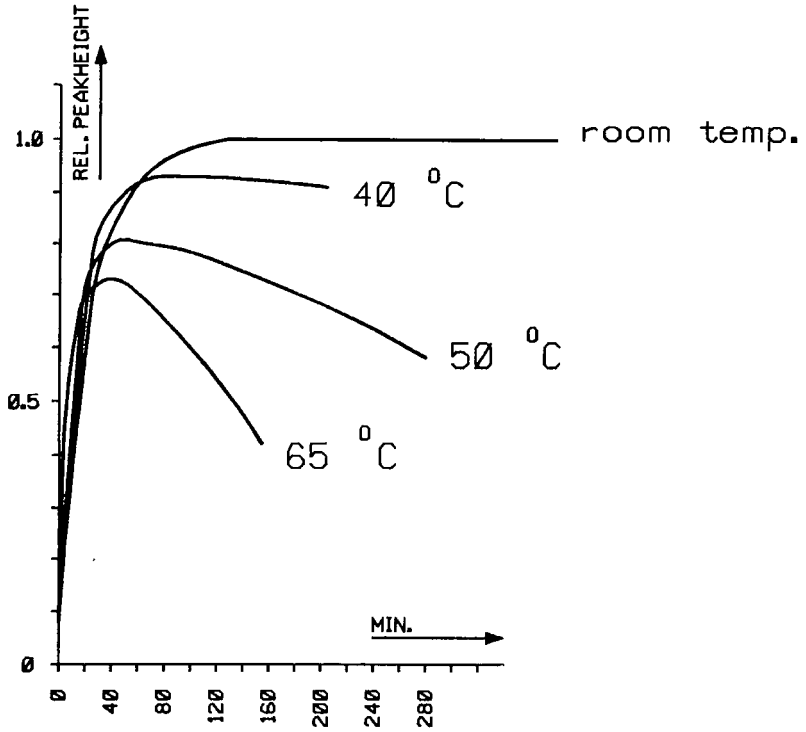

FIGURE 2 Relative peak height as a function of reaction time at different temperatures.

Reaction at elevated temperatures increases the reaction speed, but simultaneously a competing breakdown reaction of the derivative is observed. From these results it was decided to react at room temperature.

Preconcentration and clean-up

Due to the large sample size, injected onto the system, it is necessary to concentrate the compound of interest on top of the column before elution is started. In order to protect the analytical column, this is done on a small precolumn. The capacity factor of the nonenal derivative increases drastically with decreasing methanol content of

the mobile phase. At 70% methanol $k' > 30$, which offers the possibility for concentration of the derivative on reversed phase C18 material from large sample volumes.

The retention of the analyte is not only influenced by the methanol content, but also by the pH of the mobile phase. Figure 3 shows the relation between k' of the dansyl derivative and the pH of the mobile phase containing a fixed amount of methanol. The decrease of k' at pH values higher than 4 is probably due to the dissociation of the acidic proton on the nitrogen atom in the dansyl group, resulting in a charged molecule. From Figure 3 it can be estimated that the pK_a value is about 6.0, implying that the pH of the mobile phase must be 4 or lower.

From these results it was decided to use a mobile phase with pH 4.0. For practical reasons this pH was established with an acetate buffer, because phosphate buffers sometimes showed precipitation with 80% methanol. In order to measure the capacity of the precolumn for concentration of the derivative from solutions we recorded a breakthrough curve. A solution of the purified dansyl-

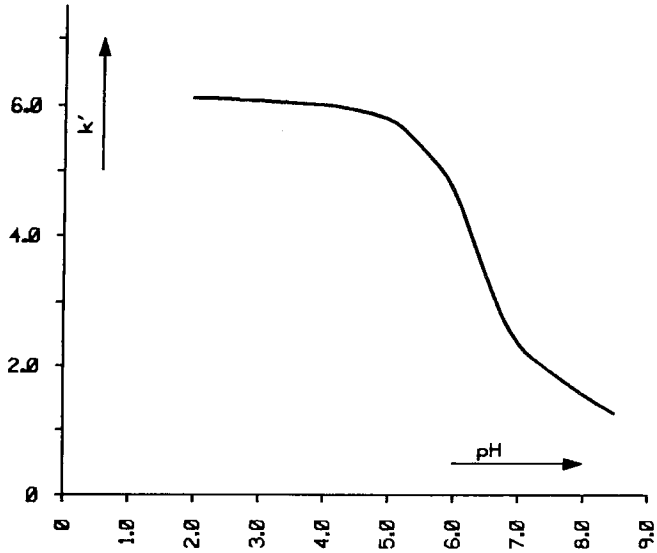

FIGURE 3 Relation between k' of dansylhydrazone of E-2-nonenal and the pH of the mobile phase containing 80% methanol.

hydrazone of E-2-nonenal ($\sim 45\,\mu g/l$) in 50% methanol in 0.05 M acetate buffer (pH 4.0) was pumped through a 10×2 mm I.D. cartridge packed with $10\,\mu m$ R Sil C_{18} D as stationary phase. By differentiation of the recorded curve the breakthrough volume, V_B, could be determined as $V_B = V_R - 3\sigma$ where V_R = retention volume and σ = standard deviation of the differential curve. The breakthrough volume for the cartridge was found to be about 12 ml (± 0.2 ml). For further experiments a maximum preconcentration volume of 12 ml was used.

Use of the described concentration with 50% methanol/0.05 M acetate buffer pH 4.0 also gives a clean-up of the sample. Excess of the reagent present in the sample is efficiently removed in this step, because it is not retained by the precolumn. This is important in order to obtain low detection limits, because dansylhydrazine itself also shows fluorescence giving a large detector signal when eluting from the analytical column.

Heartcutting

Although there is a considerable clean-up in the preconcentration step, beer samples still give a large peak, which is strongly tailing. Since the nonenal derivative elutes on this tail a sensitive detection is not possible. Improvement of separation by lowering the modifier content of the mobile phase results in a strong broadening of the peak and increased retention times. Therefore we investigate the possibilities of the transfer of the fraction containing the nonenal derivative to a second column in order to make the mass ratio of the analyte and the compounds of the tail more favourable. However, by using identical stationary phases, transfer of a fraction of column 1 to column 2 results in a severe band broadening due to the high eluting strength of the mobile phase of column 1 entering column 2. This band broadening can be overcome by applying a peak compression before starting the elution on column 2. This can be realized by decreasing the elution strength of the effluent of column 1 before it enters column 2. Mixing the eluent of column 1 with water, so decreasing the relative amount of methanol is a solution to this problem.[9]

Decreasing the percentage of methanol to 50% results in a strong increase of k' as mentioned before and thus in a concentration on the

top of column 2. The effluent was diluted by mixing with a 0.05 M acetate buffer, pumped via a modified $\frac{1}{16}''$ union tee. This T-piece and the tubing for connection with valve 4 created adequate mixing of the two solvents. Unfortunately, direct transfer of the fraction of column 1 to analytical column 2 causes rather large pressure shocks, since both analytical columns are connected in series temporarily. Therefore we tried to reconcentrate the fraction of column 1 on a small precolumn with a low pressure drop having the same dimensions as used in the first step of the chromatographic process. The precolumn was then backflushed to analytical column 2 where further separation took place.

Since the analytical column 2 was shorter and had a smaller internal diameter the transfer from column 1 to column 2 resulted in a considerable increase in peak height due to a decreased dilution (Figure 4).

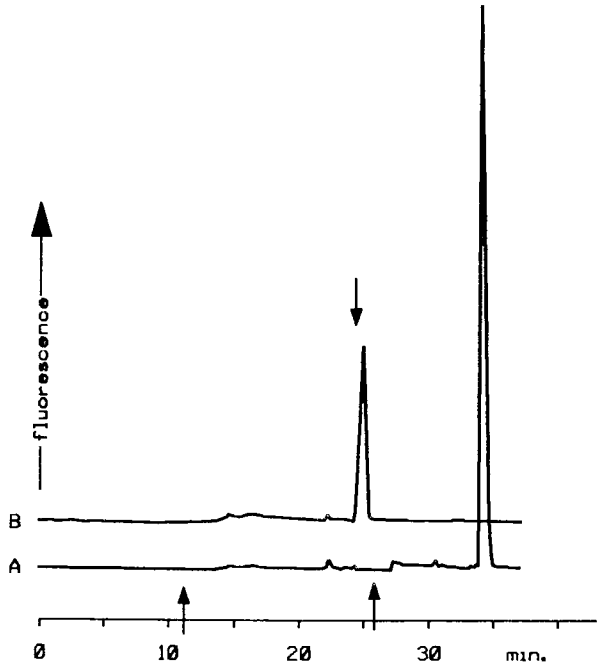

FIGURE 4 Chromatogram of dansylhydrazone of E-2-nonenal (~22 ng) with (A) and without (B) heartcutting.

Elution of analytical column 2 with methanol as the organic modifer resulted in the case of real samples in a broad lump on which the nonenal signal was superposed. Acetonitrile as modifer resulted in a much more favourable shaped plateau on which nonenal eluted (Figure 5a, b).

As stated before pH 4 gives an optimal k' value of the nonenal derivative. However, using a mobile phase with this pH to elute analytical column 1 showed an interfering peak in beer samples coeluting with nonenal. Since it is known that beer contains the saturated C9-aldehyde nonanal in a much higher concentration as E-2-N, we prepared the derivative of this aldehyde. It appeared that with a mobile phase containing 80% methanol and a pH of 4.0 E-2-N and nonanal coelute. However, with methanol/0.05 M sodium acetate 80/20 (v/v) as mobile phase, both components are base line

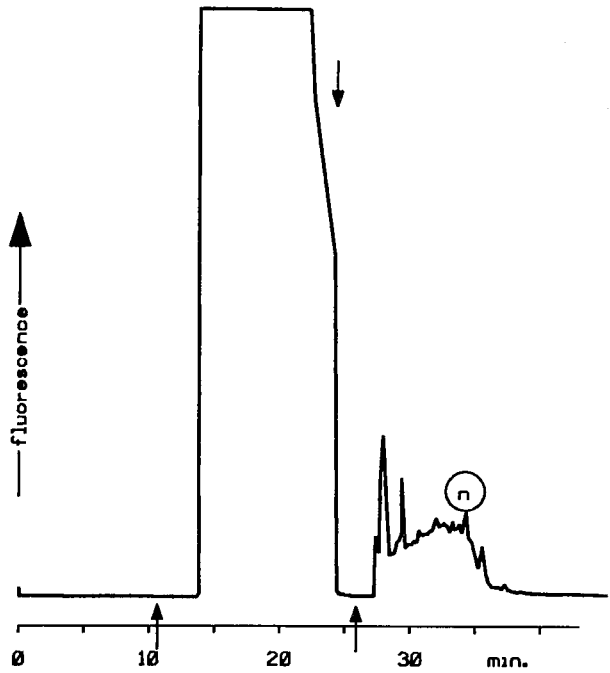

FIGURE 5a Chromatogram of fresh beer.

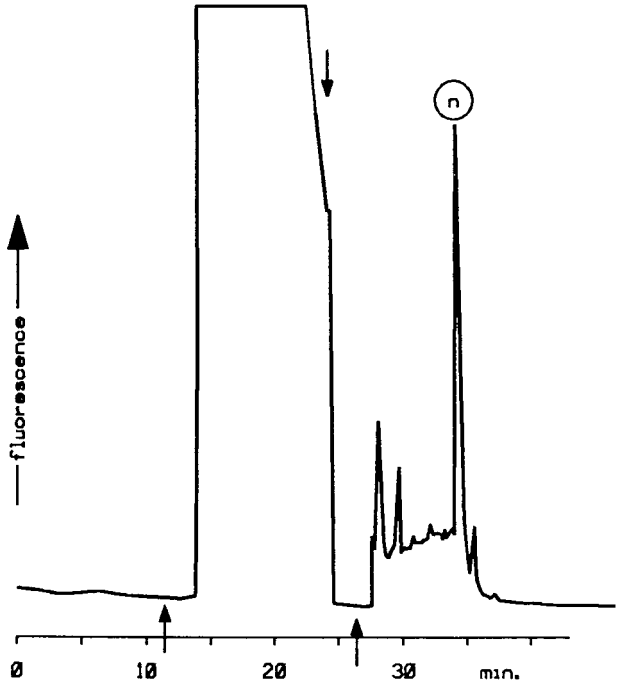

FIGURE 5b Chromatogram of beer spiked with E-2-nonenal (1 μg/l).

separated. Therefore we decided to elute analytical column 1 with a mobile phase of higher pH and analytical column 2 with a mobile phase with optimal pH 4.0. Although we do not have yet quantitative results from the described system, it is shown that analysis of E-2-nonenal at naturally occurring level is possible. Figure 6 is the chromatogram of beer naturally aged at room temperature during 8 months. The concentration of E-2-N in this beer amount to 0.26 μg/l.

CONCLUSION

This paper describes the development of the determination of E-2-N in beer at flavour threshold level (0.1 μg/l). Relative easy sample preparation gives less risk on losses or artefact formation. Using a small precolumn enables the injection of large sample volumes

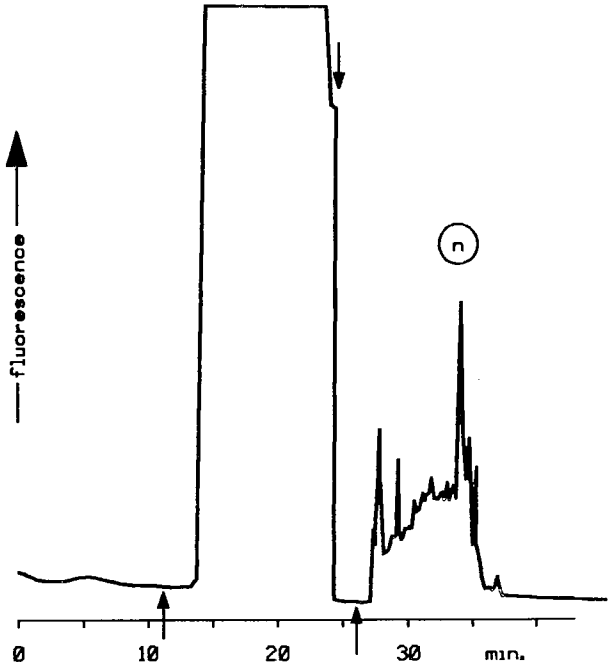

FIGURE 6 Chromatogram of beer naturally aged at room temperature.

without loss of resolution, while an off-line removal of excess of the fluorescing reagent dansylhydrazine is not needed. For quantitative results the use of an internal standard is to be preferred. Ideally this internal standard should coelute with E-2-nonenal from column 1 and be separated from it on column 2. Although the described system is still rather complicated, it has advantages above existing GC-methods as it is more suitable for automation.

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