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ANALYSIS OF *E*-2-NONENAL IN BEER AT THE ULTRA TRACE LEVEL BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING PRECOLUMN DERIVATIZATION AND COLUMN SWITCHING TECHNIQUES

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SUMMARY

A high-performance liquid chromatographic method has been developed to analyse the compound *E*-2-nonenal in beer at the ultra trace level. *E*-2-Nonenal is isolated from beer by solid-phase extraction and derivatized with dansylhydrazine. The derivative is selectively determined by a combination of on-line preconcentration and heart cutting followed by fluorescence detection. The application of boxcar chromatography enables routine analysis of *E*-2-nonenal with sample injection about every 30 min.

INTRODUCTION

Nowadays it is generally accepted that the first indication of beer staling is due to the flavour threshold of the compound *E*-2-nonenal (E-2-N) being exceeded[1–5]. Sensory evaluation has revealed that this flavour threshold is in the range of 0.05–0.1 μg/l, depending on the type of beer used in the organoleptic tests[6].

Analysis of this compound at the trace level is complicated, preconcentration and derivatization being unavoidable. Prior to derivatization, carbonyl compounds have been isolated from beer by liquid extraction[7,8] and (vacuum) distillation followed by extraction[9–11]. Most derivatization procedures involve the preparation of the 2,4-dinitrophenylhydrazones of the carbonyls which are then separated by thin-layer chromatography[11,12], gas chromatography (GC)[13] or high-performance liquid chromatography (HPLC)[7,9,10]. The GC analysis of underivatized E-2-N was developed by Strating *et al.*[14]. It involved liquid–liquid extraction and concentration by adsorption chromatography, followed by GC analysis using on-line column switching.

Major drawbacks of the above methods is that they require large samples, are very laborious and time consuming and some of them have poor resolution and sensitivity. In HPLC, the detection of carbonyls at this low concentration is possible

only as their derivatives. Several of these derivatives, UV-absorbing as well as fluorescent, and their separation by reversed-phase gradient elution have been described[15-18]. Beer is a very complicated matrix containing a wide variety of carbonyls, so extensive sample clean-up and gradient elution is needed in order to achieve separation in a reasonable time. For the analysis of a single compound in a complex sample, multicolumn chromatography or column switching is a powerful tool[19-27]. It involves the separation of multicomponent mixtures by rerouting the eluent of one chromatographic column to another, so that only a small chromatographic zone containing the peak(s) of interest is transferred to the next column for further separation. On-line operation also makes possible trace enrichment and sample clean-up with high recoveries. Recently we described the development of an HPLC system for the determination of E-2-N in beer using column switching[28]. In this paper we describe further improvements of the sample preparation, separation and quantitation of E-2-N in beer.

EXPERIMENTAL

The chromatographic system consisted of two Kratos spectroflow 400 HPLC pumps, two Milton Roy minipumps, two column-switching units (Must, Spark, The Netherlands) each with two six-port valves (Rheodyne, Model 7010). A Rheodyne Model 7125 injector was used and was provided with a sample loop with a volume of 2.3 ml. A Kratos fluorescence detector Model FS 970 was used at wavelengths of 250 nm for excitation and above 470 nm for emission using a cut-off filter. Valve switching was controlled by a microprocessor using a Model 5410 programmer (Kipp & Zonen, Delft, The Netherlands). A modified 1/16-in. Swagelok union tee was used as a mixing device. The different parts of the system are depicted in Fig. 1. The positions of the different valves are indicated by full and dashed lines.

Chemicals

Methanol and acetonitrile (HPLC grade) were from Rathburn (Walkerburn, U.K.), sodium acetate, glacial acetic acid, dipotassium hydrogenphosphate and phosphoric acid (85%) from E. Merck (Darmstadt, F.R.G.). Dansylhydrazine was obtained from Pierce and E-2-N was supplied by Ventron (Karlsruhe, F.R.G.) and used as received.

Stationary phases and columns

The precolumns used were 10 mm × 2 mm I.D. stainless-steel cartridges (Chrompack, Middelburg, The Netherlands). They were slurry packed manually with 10-μm RSil C_{18}D (Alltech-RSL, Ghent, Belgium). Analytical column 1 was 25 cm × 4.6 mm LiChrosorb 10 RP-18 (Merck). Analytical column 2 consisted of two coupled glass cartridges, 10 cm × 3 mm, packed with 5-μm Hypersil ODS (Chrompack). Sep-Pak C_{18} cartridges were from Waters Assoc. (Millford, MA, U.S.A.). The mobile phases were as follows: 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). Flow-rates of 1.0 ml/min were used with M1–M3, 0.4 ml/min with M4.
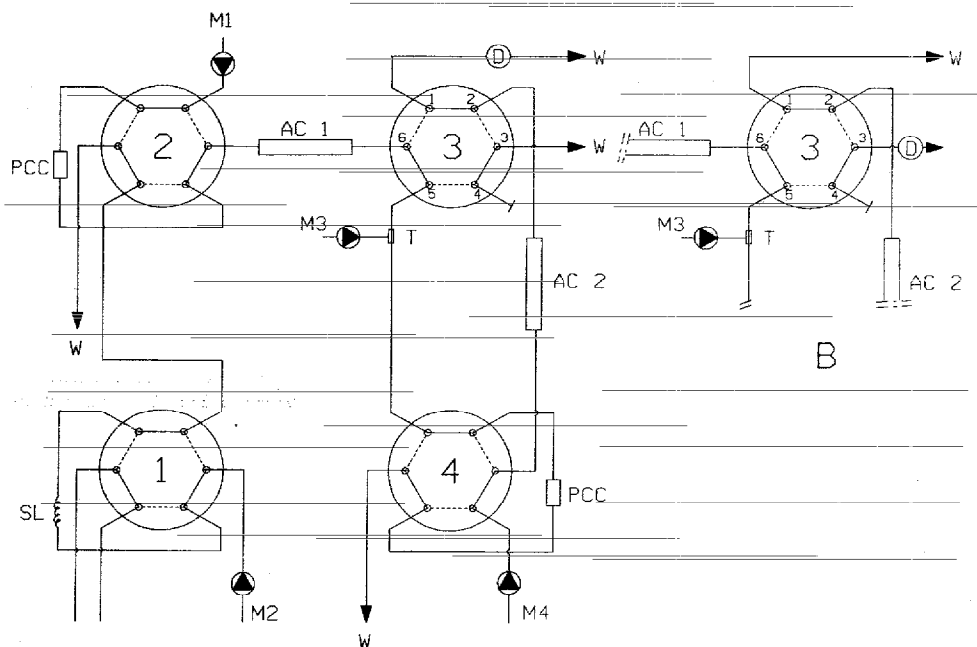
Fig. 1. Valve-switching configuration for alternate monitoring of analytical columns 1 and 2 or continuous monitoring of analytical column 2 (B). M = Mobile phase; W = waste; D = detector; AC = analytical column; T = modified union tee; PCC = preconcentration column; valve 1 = injector with sample loop (SL) of 2.3 ml. Valve-switching time schedule, after loading the injector (valve 1), for continuous monitoring of analytical column 2 (configuration B):

Time (s)	Valve 1	Valve 2	Valve 3	Valve 4
0	——	.....	.....	——
720	——	——	.....	——
735	——	.....	.....	——
1440	——	.....	——	——
1540	——	.....	.....	.....
1600	.....	.....	.....	——

Sample preparation

A 10-g amount of cooled beer (0–5°C) was passed through a preconditioned Sep-Pak C_{18} cartridge by applying a pressure of nitrogen to a small stainless-steel reservoir with a luer tip for attachment of the cartridge[14]. The cartridge was washed with 10 ml demineralized water and subsequently centrifuged for 5 min at 1000 *g*. Then the cartridge was connected upside down to a screwcap vial using a PTFE-lined septum as a 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 150 μl of 33% (v/v) acetic acid in water were added, the vial was closed and its contents were mixed. After reaction for 2 h at room temperature in the dark, the vial was stored in a refrigerator (4°C) until analysis. Immediately before analysis, the contents of the vial were mixed with 1300 μl of 0.05 *M* acetate buffer pH 4.0, and 2.3 ml of the mixture was injected into the HPLC system.

RESULTS AND DISCUSSION

The use of solid-phase extraction for the isolation of E-2-N from beer has been found to be preferable to other methods. As noted before[14], it is very difficult to concentrate the analyte by liquid–liquid extraction, due to considerable losses during evaporation of the solvent. In comparison to previous methods used for isolation of E-2-N[7,9,10], the procedure described with Sep-Pak cartridges gives savings in the number of steps and the total time. Due to the low retention of several short-chain carbonyls, carbohydrates, etc., on the C_{18} support, these compounds are removed during the concentration step. The subject of liquid-solid sample preparation has recently been reviewed[29].

It was necessary to force the beer through the cartridge by gas pressure. As beer contains carbon dioxide it is very difficult to do this manually with a syringe. On the other hand, suction of the sample through the cartridge results in much foaming. Centrifuging the cartridge removed the water hold-up, which could disturb the desorption step with methanol. On backflushing with 1 ml of methanol the adsorbed nonenal was recovered from the cartridge. Centrifuging of the cartridge connected to the vial increased the recovery. The mean recovery of E-2-N on Sep-pak C_{18} was 77% for beer spiked with E-2-N in the range of 0.1–1 $\mu g/l$. Backflushing the cartridge with a larger volume of methanol did not increase the recovery.

Although a used cartridge shows a brownish colour which can not be desorbed with methanol or hexane, experiments revealed that, by regeneration of the cartridge after each sample by flushing with 10 ml of methanol and 10 ml of water respectively, the cartridge could be re-used for at least six times without significant loss of analyte.

Derivatization

After isolation, the E-2-N in the sample was derivatized with dansylhydrazine[30,31]. Previously, we showed[28] that at room temperature this reaction was complete in about 2 h. These experiments were based on measurements at an E-2-N concentration of 17 $\mu g/l$. As natural E-2-N levels in beer do not exceed 0.2–0.3 $\mu g/l$, the reaction was re-examined at this level in model systems. Using beer samples of 10 ml means that the derivatization mixture contains 2–3 ng of nonenal in 1 ml of methanol. Therefore derivatization of E-2-N in methanol at a concentration of 2.5 ng/ml was carried out with different amounts of reagent and a fixed amount of glacial acetic acid, 50 μl per ml of methanol. With this low concentration of E-2-N the maximum peak height was reached within 2 h. Curiously, however, the maximum peak height is determined by the amount of reagent added. Fig. 2 shows a plot of the relative peak height as a function of reaction time with increasing excesses of reagent. Although the lowest amount of reagent (50 μl of 4 mg/ml solution) represents an enormous molar excess, increasing the amount of reagent resulted in an increase in peak height. The same phenomenon was noticed for *E*-2-decenal.

At first it was expected that the reagent was very quickly degraded by acetic acid. However, addition of the E-2-N 2 h after mixing of the dansylhydrazine and acetic acid showed no difference compared with direct addition of E-2-N. When dansylhydrazine was added 2 h after mixing E-2-N and acetic acid in methanol, the peak for E-2-N decreased by about 50%. Therefore it seems that E-2-N reacts in acidified methanol, possibly to the hemiacetal or acetal. As this reaction competes
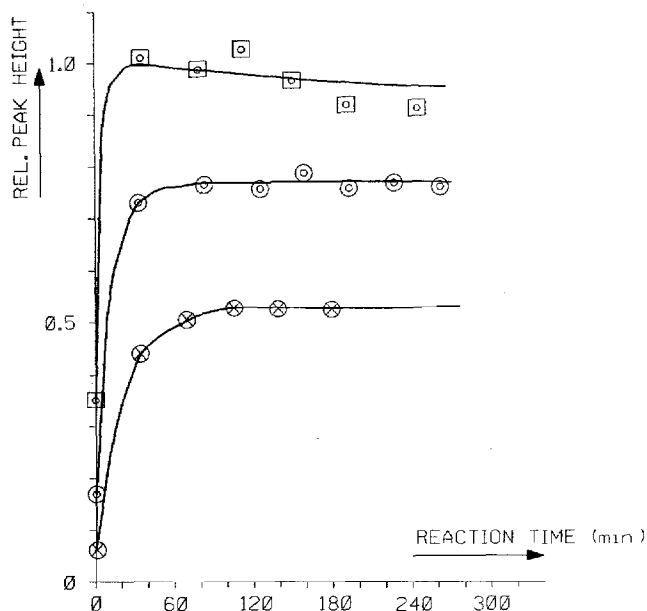
Fig. 2. Relative peak height as a function of the reaction time at room temperature for a model solution of E-2-N in methanol containing glacial acetic acid and different dansylhydrazine concentrations (□, 696; ⊙, 348; ⊗, 174 μg/ml).

with the reaction with dansylhydrazine, increasing the excess of dansylhydrazine will diminish the side reaction, thus resulting in higher peaks.

During the development of the method, it was repeatedly noticed that direct derivatization of E-2-N dissolved in pure methanol resulted in much lower peaks than those from corresponding amounts of E-2-N isolated in the described manner from spiked beer samples or a model matrix consisting of 5% ethanol, pH 4.2. As it is expected that samples prepared by the solid-phase extraction method contain small amounts of water despite the centrifugation step, we examined the influence of water on the reaction. Fig. 3 shows the relative peak height as a function of the amount of water added to the reaction mixture. It is clear that even small amounts of water have a noticeable influence on the peak height after 2 h of reaction. The maximum peak height is reached on addition of about 8% of water, while up to at least 18% has no further effect. An explanation for this phenomenon could be that acetic acid does not dissociate in methanol, which is necessary for the efficient catalysis of the reaction with dansylhydrazine. The addition of water also led to a strong reduction in the above dependence of the maximum peak height on the amount of reagent excess.

The addition of the dansylhydrazine solution 2 h after mixing the E-2-N, acetic acid and water in methanol resulted in peak heights for E-2-N comparable to those from direct addition of dansylhydrazine, indicating that the addition of water seems to diminish the above mentioned side reaction of nonenal in methanol containing glacial acetic acid. The addition of water resulted in nonenal peaks comparable to
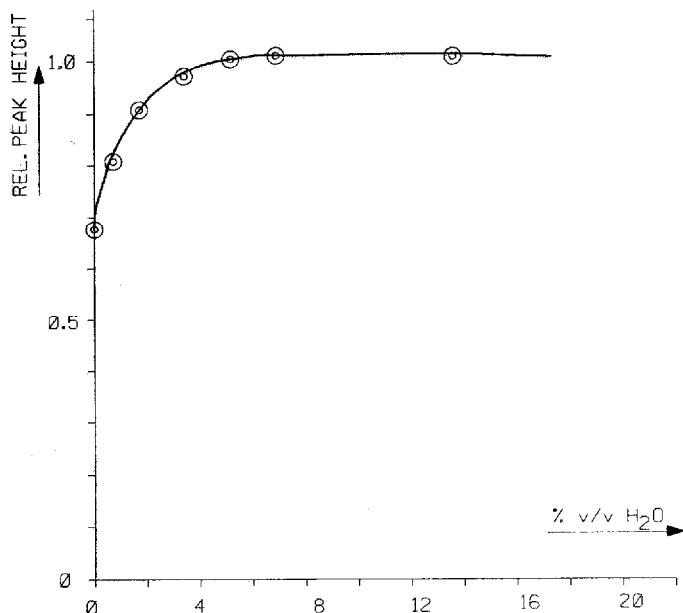
Fig. 3. Effect of the water content of the reaction mixture on the peak height of the E-2-N derivative after 2 h of reaction at room temperature.

those measured after derivatization in real samples. Although it is expected that real samples contain some water, it was decided to add a known amount of water to each sample, by adding 150 μl of 33% acetic acid in water instead of 50 μl of glacial acetic acid.

The reaction of underivatized E-2-N in methanol containing glacial acetic acid was confirmed by capillary GC coupled with a mass selective detector, using a chemically bonded fused-silica column containing a non-polar stationary phase (HP 5). A solution of E-2-N in methanol (86 mg/l) was mixed with glacial acetic acid (50 μl per ml of sample solution). Direct injection of the sample resulted in a decrease of the E-2-N peak of about 50% after 20 min at room temperature and the appearance of two new peaks with increased retention times. After 2 h at room temperature the E-2-N peak had been reduced to about 5% of its original height. The chromatogram of a sample containing aqueous acetic acid (150 μl of 33%, v/v acetic acid), however, showed no difference compared with the starting solution after about 2.5 h at room temperature. These results are in agreement with the phenomena observed during the development of the derivatization procedure for E-2-N as described above. Water seems to prevent the reaction of E-2-N with the solvent methanol which is catalyzed by acetic acid. Electron-impact (EI) mass spectra of the two peaks formed under the influence of glacial acetic acid were found to be identical, with the base peak at m/z 97 and important peaks at m/z 67, 111 and 154. These data provide support for the suggestion that the compounds are the acetal and hemiacetal of E-2-N respectively.

The sample obtained from beer after derivatization showed a slight turbidity.

On dilution of the sample in 0.05 *M* acetate buffer most of this haze disappeared. Initially we used 50 g of beer for analysis. These samples were quite turbid, even after dilution in the aqueous buffer. They were therefore filtered through a 0.5-μm filter before injection. This filtration step resulted in a ≈60% loss of the analyte. Since this was not observed when standard samples were filtrated, it is probable that the analyte was adsorbed on the turbidity.

A series of beer samples spiked with 0.5 μg/l of E-2-N were derivatized with increasing amounts of dansylhydrazine. It appeared that with increasing amounts of reagent the turbidity also increased strongly. At the same time the E-2-N peak resulting on injection of the filtrate decreased dramatically. By changing some of the chromatographic conditions it was possible to perform the analysis with 10 g of beer. With this small amount, filtration of the sample was no longer necessary. Although large numbers of samples have been injected, no blocking of the system has been observed.

Chromatographic system

The chromatographic system, with possibilities for on-line pre-concentration, cleanup and transfer of relevant fractions from column 1 to column 2 (the so-called heart cutting technique), as well as the mobile phases were previously described in detail[28]. The mobile phase on column 2 is a mixture of acetonitrile and acetate buffer pH 4.0. It was previously shown that the capacity factor, k', of the derivative of E-2-N is dependent on the pH of the mobile phase. Also the fluorescence intensity is influenced by the pH. Fig. 4 shows that the peak area is dependent on the pH of the mobile phase. The optimum fluorescence intensity was measured at a pH of about
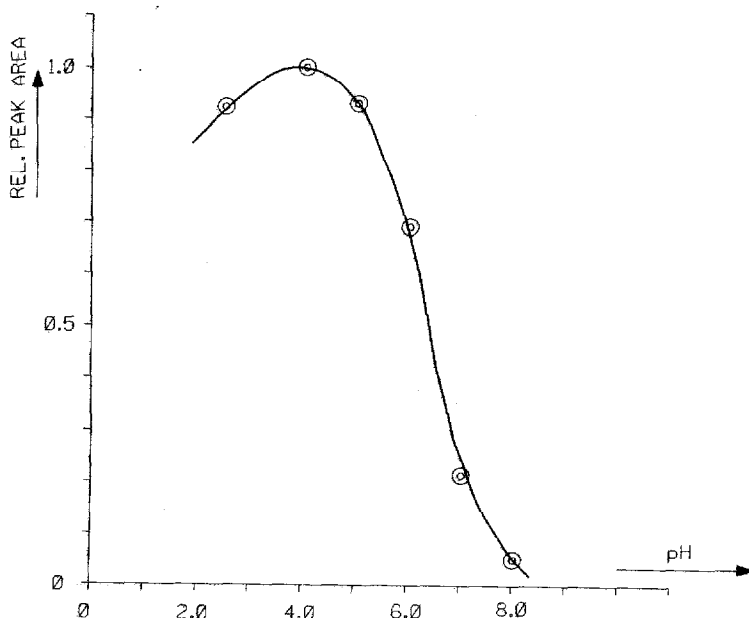

Fig. 4. Relative peak area of the dansylhydrazone of E-2-N as a function of the pH of the mobile phase containing 80% methanol and 20% phosphate buffer (0.05 *M*).

4.0. It is expected that with increasing pH the molecule becomes charged by disso-
ciation of the acidic proton on the nitrogen atom in the dansyl group, thus resulting
in decreased fluorescence intensity.

For the separation on analytical column 2 several C_{18} supports have been
tested. Hypersil ODS gave the best results. Spherisorb ODS gave a very broad lump
with the E-2-N derivative superposed on it. Chromspher ODS yielded much smaller
k' values. Using as analytical column 2 a column with internal diameter 3 mm op-
erated at 0.4 ml/min, a considerable gain in peak height was obtained due to de-
creased dilution and the lower flow-rate through the detector in a mobile phase with
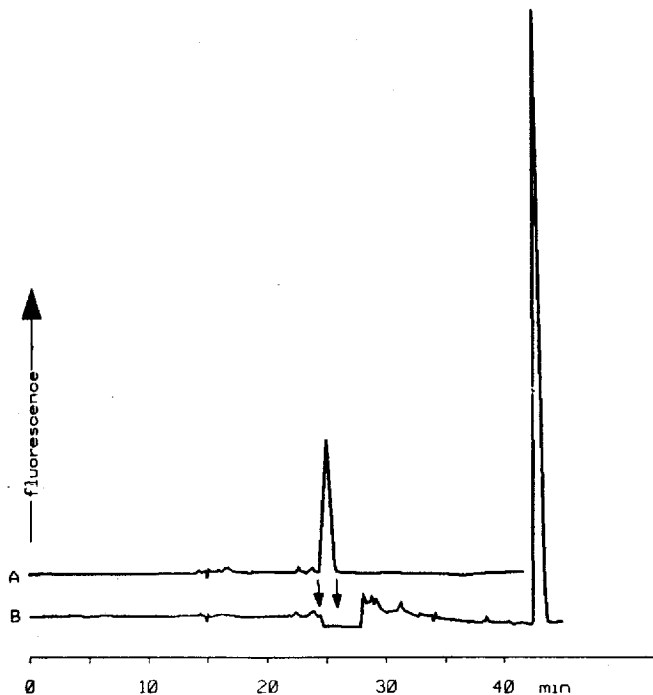optimum pH, as demonstrated in Fig. 5.

Fig. 5. Chromatogram of the dansylhydrazone of E-2-N ($\approx$ 5 ng) eluted from analytical column 1 (A)
and after heart cutting, eluted from analytical column 2 (B).

The reduction in the amount of beer used for analysis to 10 g also reduced the
large front peak eluted from the analytical column 1, Fig. 6. Although decreasing the
amount of beer resulted in less tailing, the E-2-N was still eluted as a very small peak
on the tail of this front peak. Further attempts to reduce the front peak by cleanup
on the Sep-Pak cartridge were not successful. Flushing the cartridge with water–
methanol mixtures up to 50% methanol did not decrease the front peak.

Transfer of the fraction containing E-2-N eluted from column 1 and separation
on column 2, after reconcentration on precolumn 2, results in a much better chro-
matogram. The chromatogram from analytical column 2 is a plateau with E-2-N
eluted as a distinct peak. The insert to Fig. 6 shows the chromatogram of the fraction
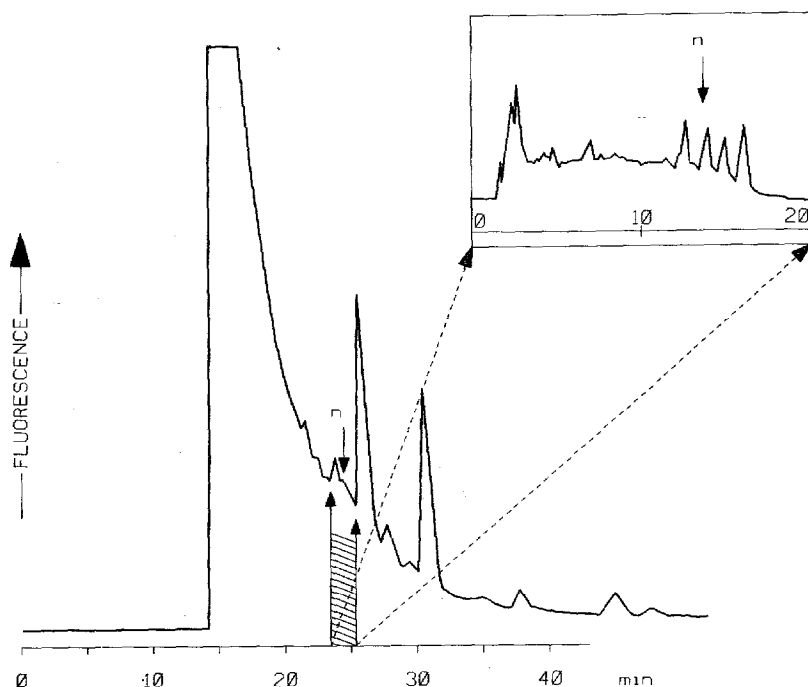from 24 to 26 min obtained by monitoring of analytical column 2.

Fig. 6. Chromatogram of beer obtained from analytical column 1. E-2-N is indicated by an arrow in the dashed zone. Insert: chromatogram of heart cut from 24–26 min obtained by monitoring analytical column 2.

To determine that fraction from column 1 that has to be transferred, a partly purified nonenal derivative was injected and its elution monitored on column 1. It was found that a transfer time of 100 s was satisfactory. Increasing the transfer time did not improve the chromatogram from column 2, but gave more peaks eluted before or after the E-2-N peak.

As it is very important to control the retention time on column 1, attention must be paid to the preparation of the mobile phase. It was noticed that reproducible mixing of the methanol aqueous buffer was critical. This was best achieved by weighing both components. Because there were still slight changes in the retention time of the analyte on column 1 between different batches of mobile phase, it was practical to determine the retention time of the nonenal derivative once a day or after replenishing of the mobile phase, by injecting a solution of the partly purified derivative of E-2-N.

Although a single experiment takes about 50 min, it is not necessary to monitor the eluent of column 1 continuously, so after the determination of the switching times the tubing of ports 1 and 3 of valve 3 were simply interchanged (Fig. 1b), thus allowing continuous monitoring of analytical column 2. This made it possible to double the sample throughput. As soon as the first sample had reached column 2 the second sample could be injected into column 1. Fig. 7 shows the results for six samples injected at about 30 min intervals. This application of boxcar chromatography[32] is very attractive for routine application. In order to contaminate the first analytical
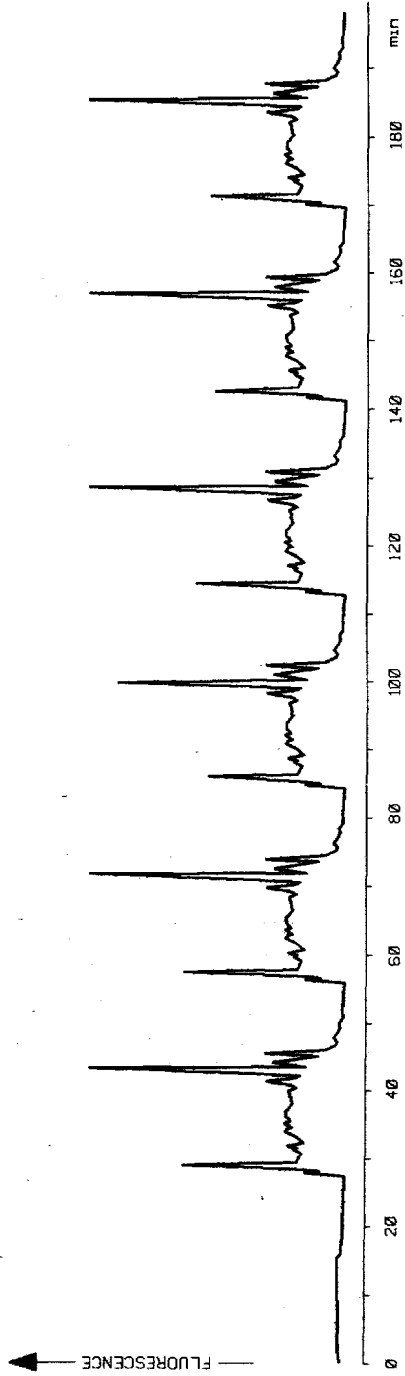
Fig. 7. Chromatograms of six beer samples spiked with E-2-N to 0.5 μg/l and injected at about 30-min intervals (boxcar chromatography), obtained by continuous monitoring of the eluent from analytical column 2.

column as little as possible with strongly retained components, backflushing of the preconcentration column 1 to analytical column 1 was optimized. Experiments showed that 15 s of backflushing are enough to transport the analyte completely to the analytical column 1. Immediately after backflushing, precolumn 1 was clean by injection of one loop volume (2.3 ml) of methanol. Both precolumns were repacked each day.

Calibration curves for E-2-N were constructed in the concentration range of 0.1–1 μg/l by analyzing beer spiked with known amounts of E-2-N. This resulted in a linear calibration plot, $y = 166.7x + 2.5$, were x = concentration (μg/l) and y = peak height, with a regression coefficient of 0.9990. Repeated analysis of a beer sample containing 0.2 μg/l of E-2-N gave a relative standard deviation of 8.2% ($n = 9$). The mean overall recovery of the method, determined as the ratio of the slopes of the calibration plots obtained from spiked beer and from injection of directly derivatized amounts of E-2-N, was found to be 78%. As described earlier, the recovery from the Sep-Pak isolation step was 77%, suggesting that the loss of E-2-N occurs mainly in the isolation step. The limit of detection of E-2-N was 0.2 ng. The limit of determination is 0.02 μg/l.

Fig. 8 shows the chromatogram of a beer containing an E-2-N content of 0.11 μg/l and the same beer after heating at 40°C for 4 days, resulting in an E-2-N concentration of 0.36 μg/l.
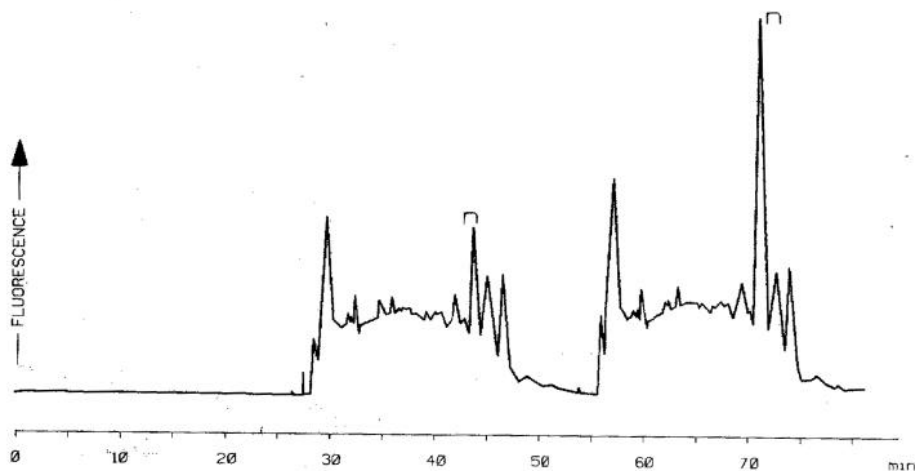

Fig. 8. Chromatogram of fresh beer (left) and after heating for 4 days at 40°C.

CONCLUSIONS

The method described for selective determination of E-2-N in beer at the ultra trace level offers advantages over previously reported methods with respect to sample preparation and the amount of sample needed for analysis. The combination of solid-phase extraction, precolumn derivatization and the application of column switching (boxcar) chromatography enables routine analysis at trace level with relatively small samples, with sample injection about every 30 min. As the transfer of the nonenal-containing fraction from column 1 to column 2 is critical, it is absolutely

necessary to have a stable retention time of the analyte on column 1. This places high demands on the pump used to elute analytical column 1. By using time-programmable switching valves, the system can easily be automated for unattended operation.

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