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Determination of *E*-2-nonenal by high-performance liquid chromatography with UV detection Assay for the evaluation of beer ageing

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Abstract

The analysis of *E*-2-nonenal is of considerable interest for the brewery industry as this compound is claimed to be responsible for a paper/cardboard unpleasant flavour. Usually, the presence of *E*-2-nonenal can be noticed in aged beers at levels higher than 0.1 μg/l. In this work, an analytical method was developed to determine *E*-2-nonenal in beer involving steam distillation of beer followed by an extraction/concentration step using solid-phase extraction and determination of *E*-2-nonenal by HPLC with UV detection. Fastness and simplicity are the main advantages of the proposed method, when compared with other existing methodologies for the determination of *E*-2-nonenal in beer. Using the developed conditions, the interference of *E*-2-nonenal formed by degradation of its precursors during steam distillation is almost negligible. The presence of sulphur dioxide at legal levels does not interfere with the assay. The method was used in a comparative study of fresh and either naturally or forced aged beers. A much larger chromatographic peak was found near the peak of *E*-2-nonenal that correlates well with the peak of *E*-2-nonenal. Identification of the corresponding compound is currently under investigation, considering its future application on the evaluation of beer ageing.

Keywords: Beer; Food analysis; Nonenal; Carbonyl compounds

1. Introduction

Beer flavour stability has always been a concern to the brewing industry, especially when beer is stored at high temperatures. It is well-known that carbonyl compounds formed and/or released during storage of packaged beer, particularly unsaturated long chain aldehydes with a very low taste/odour threshold, are responsible for organoleptic defects found in beer as it ages. In appropriate concentrations some aldehydes could generate pleasant flavours; however, at higher levels, they are responsible for the development of an oxidized odour/flavour [1].

E-2-Nonenal has received particular attention as the major source of the oxidized flavour. This aldehyde has a very low threshold level (in the range of 0.05–0.1 μg/l) and at higher levels is responsible for the paper/cardboard character developed in aged beers [2]. Although this characteristic flavour is typical in aged beers, from time to time it can be noticed in beers with only 3 months of storage.

The pathways that explain the formation of *E*-2-nonenal during beer storage are still unclear. Some authors mentioned that *E*-2-nonenal forms adducts with beer constituents like sulphur dioxide [3,4], and

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amino acids [5], which could be disrupted as beer ages. Other authors pointed out that the increase in *E*-2-nonenal concentration might be, essentially, due to its formation from precursors. In this context several hypotheses can be found in the literature: *Strecker* degradation of amino acids [6], oxidative degradation of isohumulones [7], oxidation of fatty acids [2,8] or aldol condensations [9]. In addition, it is known that beer flavour stability is highly dependent on storage temperature [9–11], pH [12] and oxygen level [2,8].

The presence of sulphur dioxide in beer is another factor that has a marked influence on beer ageing. It is well-known that its concentration gradually decreases during storage and the explanations of such behaviour are today well understood. Sulphur dioxide protects beer against oxidation, particularly from oxygen reactive species. Additionally, it has another important role in beer by forming adducts with carbonyl compounds, increasing their taste/odour threshold. Although some sulphur dioxide occurs in beer naturally, an extra addition of the compound is generally made to increase the protective effects just mentioned.

Because of the unusually trace level of *E*-2-nonenal in beer, analysis of this compound is complicated. Most procedures involve preconcentration followed by a derivatisation step. Carbonyl compounds are isolated from beer by liquid–liquid extraction [13,14] and vacuum distillation [15,16]. 2,4-Dinitrophenylhydrazine is the most widely used derivatising agent [13,15], but dansylhydrazine [17] and dabsylhydrazine [18] are preferred, because of their high sensitivity. These aldehyde derivatives are then separated and estimated by HPLC. GC–MS analysis of carbonyl compounds derivatised to corresponding thiazolidines with cysteamine was recently reported by Yasuhara et al. [1], although underivatised *E*-2-nonenal can be directly detected [14,16].

Most of the above mentioned methods are laborious and time-consuming, requiring strong acidic conditions to obtain derivatives, which may alter the carbonyl compounds of interest or produce additional carbonyl compounds. On the contrary, the method reported here is easier with no need for derivatisation. As previously reported for 4-hydroxynonenal in biological samples [19] we believe that the 226 nm UV-absorption maximum of *E*-2-nonenal is more sensitive than the gas chromatographic–mass spectrometric method. Beer is a very complicated matrix containing a wide variety of compounds absorbing in the 226 nm range of the aldehyde. It is therefore clear that prior to HPLC analysis an effective sample clean-up as well as a concentration step is essential. This paper describes every step involved in the analysis as well as an application of the developed method for the monitoring of *E*-2-nonenal during natural and forced ageing.

2. Materials and methods

2.1. Instrumentation

A Gilson HPLC instrument was used, consisting of a 307 pump, a 115 UV variable wavelength detector, selected to 226 nm, a Rheodyne 7125 injection valve with a 20-μl loop and a Spectra Physics DataJet CH1 integrator. A precolumn Nucleosil ODS (8 mm×4 mm, 5 μm particle size) and a column Nucleosil ODS (250 mm×4 mm, 5 μm particle size) from Macherey–Nagel were used. Isocratic elution was used, with a flow-rate of 1.00 ml/min. A water–acetonitrile (45:55, v/v) mixture was used as mobile phase. The mobile phase was filtered and degassed in a vacuum filter holder, Schleicher & Schuell GV 050/0, with 0.2 μm Schleicher & Schuell NL 16 membrane filters.

Solid-phase extraction (SPE) columns of 200 mg Chromabond C_{18} were from Macherey–Nagel. A steam distillation system was used, made with ordinary laboratory glass materials. A PTFE tube was used to connect the steam generator vessel with the sample vessel.

2.2. Chemicals

Acetonitrile (HPLC grade) and sodium chloride (analytical reagent grade) were purchased from Merck. Potassium disulfite (analytical reagent grade) was obtained from Riedel-de Haën. *E*-2-Nonenal (analytical reagent grade) was from Aldrich. The water used to prepare all solutions was deionised, distilled and further purified in a Simplicity Millipore system. Stock solutions of *E*-2-nonenal were pre-

pared weekly in water–acetonitrile (50:50, v/v). In this medium, *E*-2-nonenal is soluble and stable.

2.3. Experimental procedure

The methodology developed to extract and quantify *E*-2-nonenal is divided into three main steps: (1) steam distillation of a sample solution; (2) passage of the distillate through an SPE column (*E*-2-nonenal is retained in the column); (3) elution and analysis by HPLC with UV detection. Before the distillation step, 2.9 g of sodium chloride are added to 250 ml of 6% (v/v) ethanol solutions of *E*-2-nonenal. Fifteen ml of distillate are collected, diluted with water to 100 ml and passed through the SPE column. *E*-2-Nonenal retained in the SPE column is extracted with 1.00 ml acetonitrile and injected in the HPLC column. The same conditions are used in the analysis of beer, replacing the 6% ethanol model solutions by the beer samples.

3. Results and discussion

3.1. Development of the method of analysis of E-2-nonenal

3.1.1. Extraction/concentration step

The steam distillation of solutions of *E*-2-nonenal was investigated using an aqueous solvent containing 6% (v/v) of ethanol. As can be seen in Fig. 1, the extraction of *E*-2-nonenal is completed when 10% of the initial volume is distilled.

The effect of the alcoholic content of the distillate on the retention of *E*-2-nonenal at the SPE column was also studied. As it was expected, lower recoveries of *E*-2-nonenal are obtained for higher ethanol contents. Indeed, since ethanol is less polar than water, it favours the elution of *E*-2-nonenal during the passage of the sample through the SPE column. This was confirmed by the results presented in Fig. 2, showing that the retention of *E*-2-nonenal at the solid-phase is drastically reduced if the ethanol concentration of the distillate is higher than 30%. Since the volume of distillate collected is only about 10% of the volume subjected to distillation and ethanol has a lower boiling point than water, the concentration of ethanol in the distillate can be higher than 30%. To avoid this problem, the distillate must be diluted with water before being passed through the SPE column.

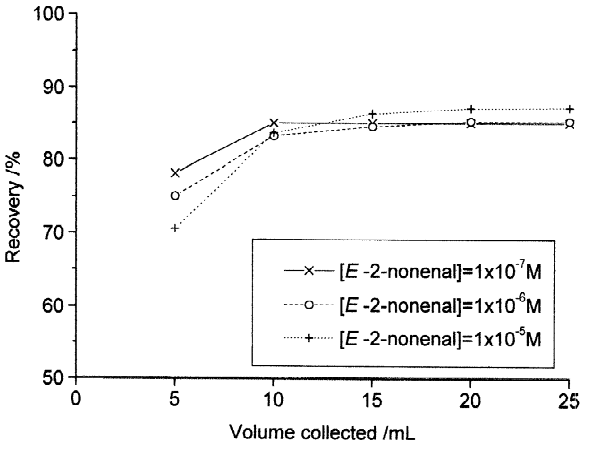

Fig. 1. Recovery of *E*-2-nonenal during steam distillation. Volume distilled, 100.0 ml.

E-2-Nonenal was extracted from the SPE column with 1.00 ml of acetonitrile and 20.00 μl of the extract were injected into the HPLC column. A good separation and quantification of the peak of *E*-2-nonenal was obtained using a water–acetonitrile (45:55, v/v) mobile phase and a flow-rate of 1.00 ml/min. With the optimised conditions reported in Section 2.3, the following relationship was obtained for 6% ethanol solutions:

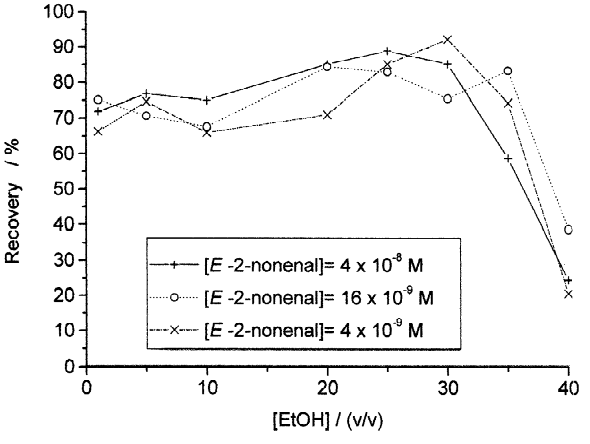

Fig. 2. Recovery of *E*-2-nonenal at SPE columns from model solutions with different ethanol concentration. Elution of *E*-2-nonenal with 1.00 ml of acetonitrile.

peak area = 7.98[*E*-2-nonenal] (mol/l) $\times 10^9 - 105$

3.1.2. Interference of E-2-nonenal precursors present in beer

Another problem considered was the possibility of generation of extra *E*-2-nonenal during the steam distillation step. In fact it is well-known that high temperatures accelerate beer ageing and promote the formation of *E*-2-nonenal from its precursors. To try to obtain an answer to this problem, samples of beer were subjected to different distillation conditions.

As can be seen in Fig. 3, the amount of *E*-2-nonenal formed from its precursors during the steam distillation of beer is highly dependent on pH.

The strong increase on *E*-2-nonenal formation at lower pH values was already described by Stenroos et al. [20]. Distilling beer under reflux at pH 2 these authors obtained *E*-2-nonenal concentrations more than 100 times higher than the perception limit and suggested that this increase was due to the degradation of the acids 9,12,13-trihydroxy-10-octadedecenoic, 9,10,13-trihydroxy-11-octadedecenoic and 9,10,11-trihydroxy-12-octadedecenoic. No explanation was discovered for the slight increase in *E*-2-nonenal concentration at higher values of pH. Therefore, it is recommended to use beer without any pH adjustment (pH 4–4.5).

Even at the pH of beer, if the sample is heated during longer periods of time under reflux there is a continuous production of *E*-2-nonenal as can be seen in Fig. 4. It is worth noting that there are two other peaks that also increase, adjacent to the *E*-2-nonenal peak. It seems therefore that these compounds could also be involved in beer ageing, a subject that will be returned to later on in this discussion.

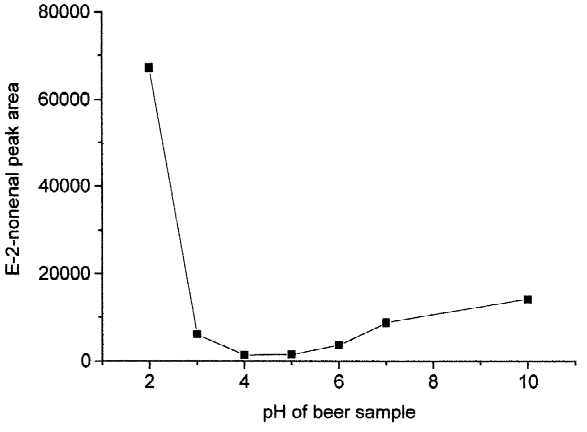

Fig. 3. Effect of the pH of beer on the amount of *E*-2-nonenal obtained by steam distillation.

In view of the results obtained, a more specific study was performed to evaluate the production of *E*-2-nonenal during the distillation step. Aliquots of a beer sample (250.0 ml) were analysed, collecting different volumes of distillate and determining the *E*-2-nonenal concentration using the developed methodology. The study was repeated several times with different beer samples; in one of the experiments beer was spiked with *E*-2-nonenal. As Fig. 5 shows, if a volume of distillate lower than 15 ml is collected from a sample of 250 ml of beer, the formation of *E*-2-nonenal is negligible and does not affect the results. In fact, a clear levelling of the signal is obtained for volumes of distillate between 10 ml and 15 ml, which means that within this volume range the recovery of *E*-2-nonenal is practically independent of the volume of distillate collected (all the *E*-2-nonenal is already extracted and its production from precursors is negligible).

In conclusion, if a sample of 250 ml of beer is analysed, 15 ml seems to be a convenient volume of distillate to be collected: it is sufficient for the extraction of *E*-2-nonenal from beer and it is small enough to avoid a significant generation of *E*-2-nonenal from its precursors. This was confirmed by the fact that no *E*-2-nonenal was found on a second fraction of 15 ml of distillate, collected after the distillation of the first 15 ml.

3.1.3. Interference of sulphur dioxide

As it was already mentioned, sulphur dioxide forms reversible adducts with aldehydes, including *E*-2-nonenal. Therefore, it was decided to study the influence of sulphur dioxide on the distillation of beer samples. The investigation was carried out on beer samples spiked with 0, 10, 20 and 30 ppm of sulphur dioxide, respectively, and no interference was observed for values up to 20 ppm. Since sulphur dioxide concentration in beer never exceeds 20 ppm, it can be concluded that this compound does not interfere in the distillation and so does not affect the methodology proposed.

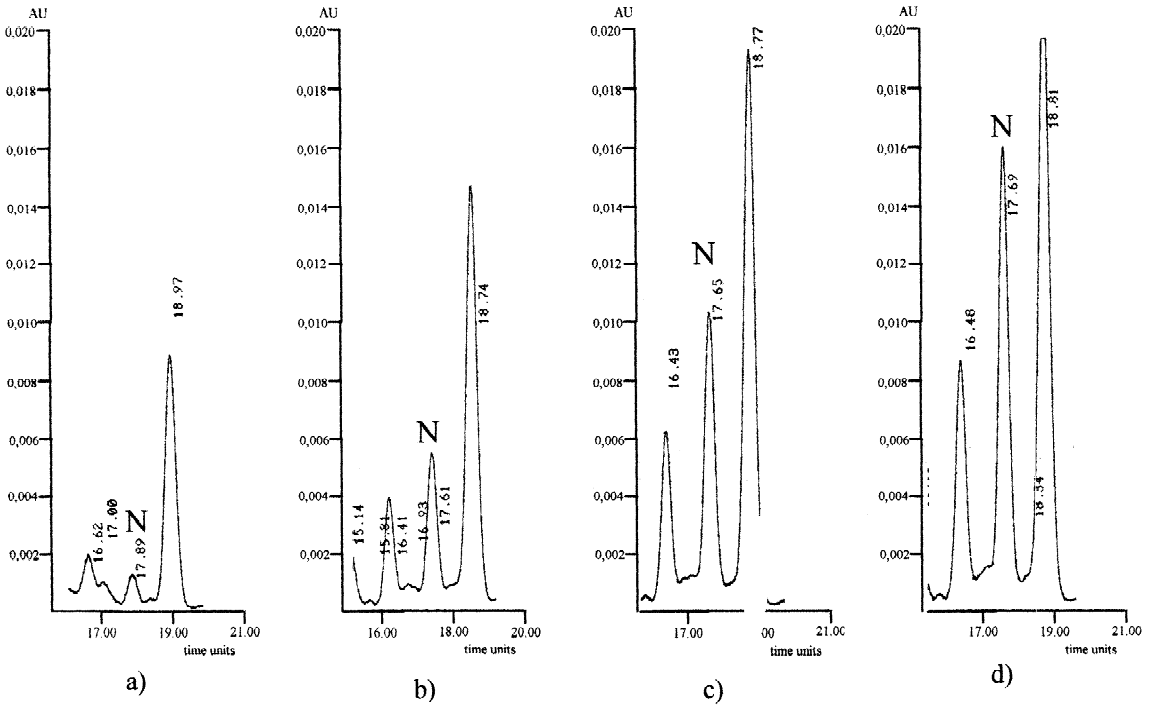

Fig. 4. Chromatograms illustrating the effect of heating time (under reflux) on the production of *E*-2-nonenal in beer. Heating time, before distillation (h): (a) 0; (b) 1; (c) 2; (d) 3. N, *E*-2-nonenal peak.

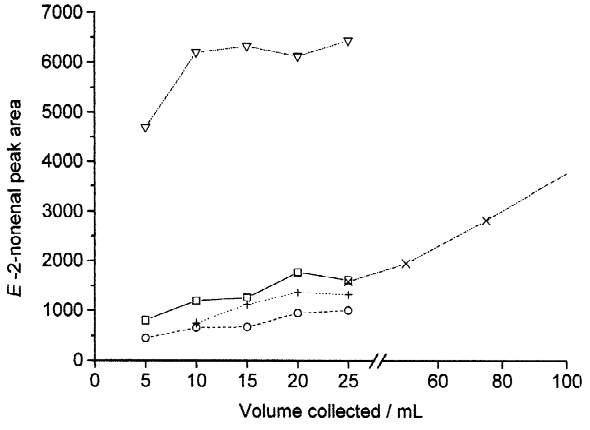

Fig. 5. Peak area of *E*-2-nonenal as a function of the volume collected during steam distillation of 250 ml of beer: ○, +, ×, □, beer samples; ▽, beer sample spiked with *E*-2-nonenal.

3.2. Determination of E-2-nonenal in beer

A typical chromatogram of a beer extract is illustrated in Fig. 6. In spite of the very low concentration of *E*-2-nonenal usually present in beer, a peak for the compound is clearly seen and can be used for quantification purposes.

It is important to note that in the determination of *E*-2-nonenal in beer a compensation for losses of the compound during the extraction/concentration step must be considered. In fact, results of Table 1 show a recovery of only 40 to 50% for different amounts of *E*-2-nonenal added to beer samples before distillation. This problem of low recovery of *E*-2-nonenal was solved using the method of standard additions, with the standards of *E*-2-nonenal being added to beer before distillation. The results obtained in the determination of *E*-2-nonenal using the proposed method are within the range usually found in beer (slightly below 0.10 mg/l).

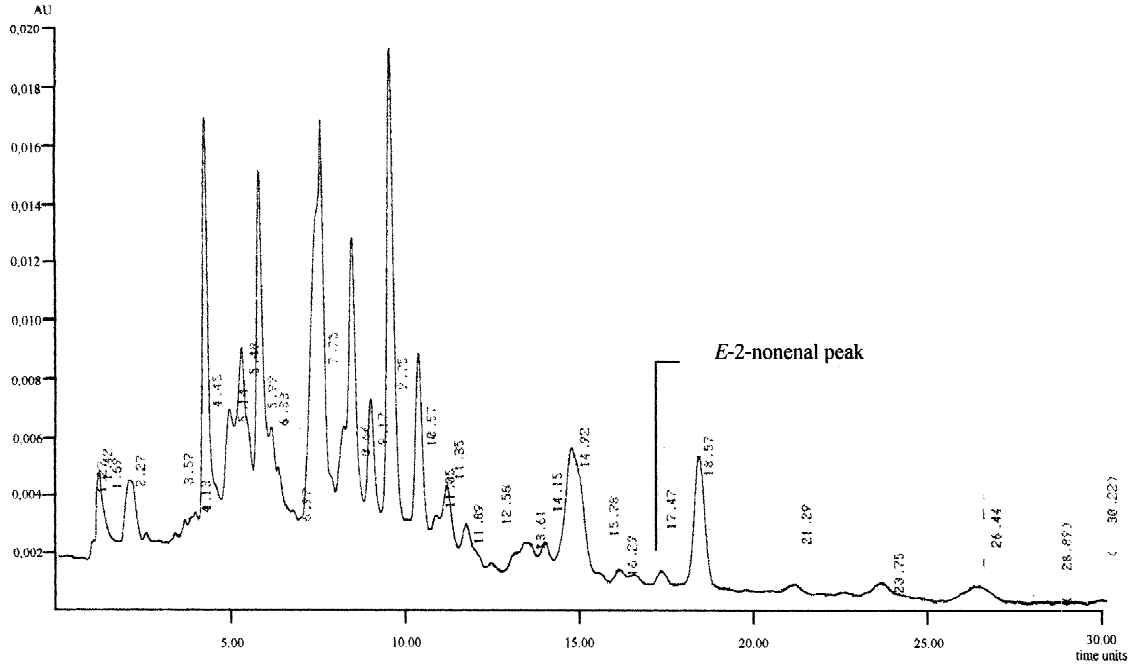

Fig. 6. Typical chromatogram of a beer extract.

3.3. Determination of E-2-nonenal in beer subjected to forced ageing

A forced ageing assay is used in the beer industry as a long-term prediction test for assessing beer flavour stability. In this assay beer is maintained at 37 °C for 7 days. It is accepted that these conditions are equivalent to an ageing period of 6 months at 20 °C. The profile of *E*-2-nonenal observed under forced ageing is described by some investigators [2,11]. In this work the forced ageing assay was used to assess the validity of the proposed methodology. Several bottles of fresh beer were stored at 37 °C and analysed in duplicate within 14 days. The results obtained can be seen in Fig. 7. Results show that the concentration of *E*-2-nonenal in beer increases during the first 5 to 10 days of storage at 37 °C and almost levels during the last days.

In order to compare natural and forced ageing, another experiment was conducted. Bottled beer was analysed fresh and then stored in the dark at room temperature for 9 months (natural ageing) and 37 °C for 1 week (forced ageing). Typical chromatograms can be seen in Fig. 8. The average values obtained in the analysis of two samples of beer were 0.06, 0.14 and 0.15 μg/l, respectively, for fresh, natural and

Table 1
Recovery of *E*-2-nonenal in the extraction/concentration process

Assay	Beer spiked with *E*-2-nonenal			Direct injection of *E*-2-nonenal			Recovery ($\Delta A/\Delta B$)
	E-2-Nonenal (10^{-7} mol/l)	Peak area	Variation of peak area (ΔA)	*E*-2-Nonenal (10^{-7} mol/l)	Peak area	Variation of peak area (ΔB)	
1	x (beer)	1132	–	0	0	–	–
2	$x + 1.00$	1786	654	1.00	1380	1380	47%
3	$x + 2.00$	2108	976	2.00	2468	2468	40%
4	$x + 4.00$	3166	2034	4.00	4354	4354	47%

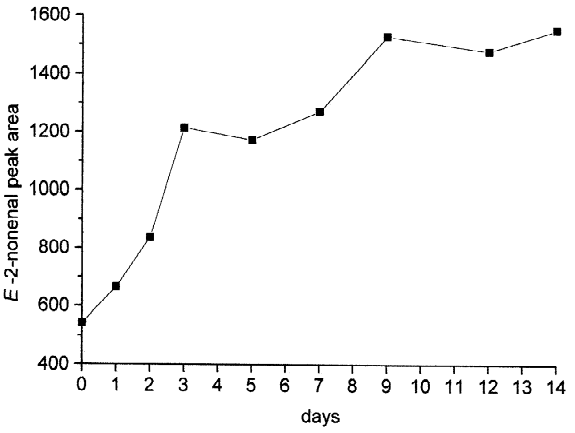

Fig. 7. Variation of the concentration of *E*-2-nonenal with the time of storage of beer at 37 °C.

forced aged beer. This behaviour correlates well with the one observed by the investigators just mentioned. It is important to note that during the storage period the concentration of *E*-2-nonenal became higher than its flavour threshold (around 0.1 μg/l).

Finally, it is worth noting that there is a peak at the right of the peak of *E*-2-nonenal (Fig. 8) that seems to show a clear dependence on beer ageing too. As the peak is much larger than that of *E*-2-nonenal it would be much easier to use it in the analytical assessment of beer ageing. Further studies are being conducted in order to identify the compound responsible for that peak. For instance, it is probably a carbonyl compound as the addition of hydrazine suppresses both the peak of the unknown compound and the peak of *E*-2-nonenal (formation of a hydrazone).

4. Conclusions

A new methodology was developed for the determination of *E*-2-nonenal in beer. The interference of *E*-2-nonenal formed by degradation of its precursors during steam distillation was found to be pH-dependent, with a minimum at the pH of beer. Heating conditions for the steam distillation of beer were established with an almost negligible formation of *E*-2-nonenal from its precursors. At the levels

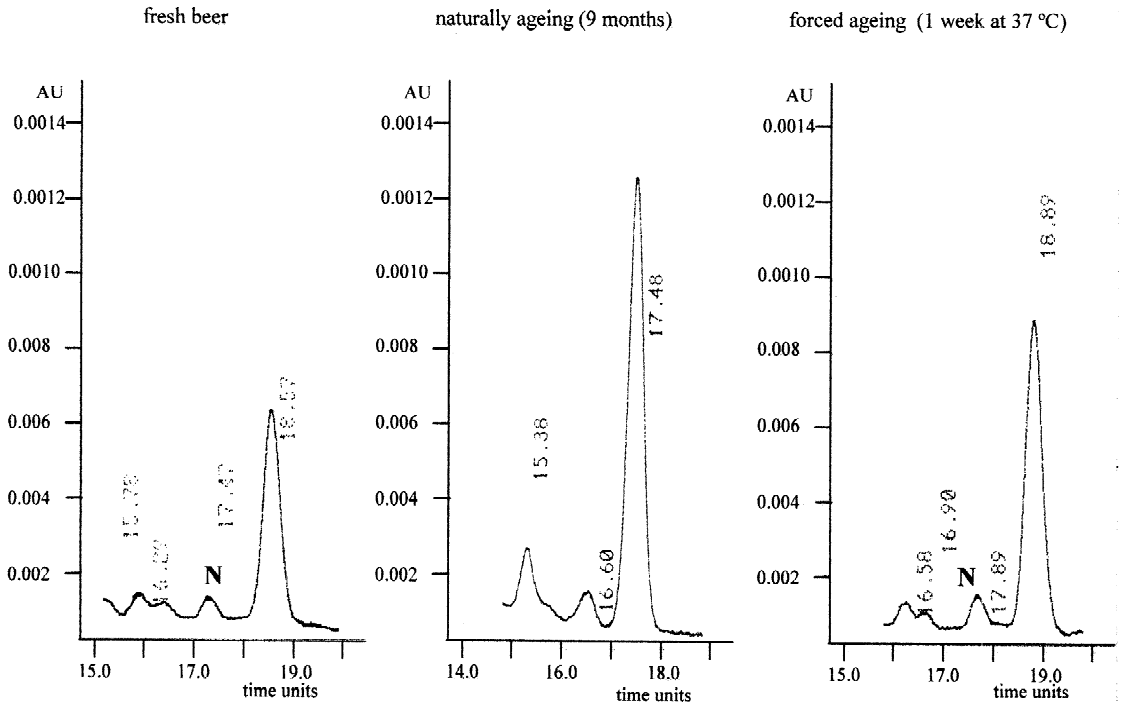

Fig. 8. Analysis of beer subjected to different ageing conditions. N, *E*-2-nonenal peak.

usually present in beer, sulphur dioxide was found not to interfere in the determination of *E*-2-nonenal.

Recovery studies performed on beer samples spiked with *E*-2-nonenal have shown that more than 50% of the compound is lost during the pretreatment of the sample. Using the standard additions method, a linear correlation was obtained between peak area and the amount of standard added. Because standard additions were made before distillation, there was a compensation for the loss of *E*-2-nonenal during sample pretreatment.

When compared with other existing methods, the major advantages of the proposed methodology are fastness and simplicity, only requiring a steam distillation, an SPE extraction and an isocratic HPLC–UV detection. The major drawback is the possibility of some conversion of precursors into *E*-2-nonenal during the steam distillation step. This fact prevents the application of the method to samples containing large amounts of *E*-2-nonenal precursors, as in the case of wort.

Finally, it is important to mention that some preliminary work indicates that a correlation seems to exist between beer ageing and a compound, not yet identified, that produces a much higher signal than *E*-2-nonenal. This is an interesting topic to be further investigated.

Acknowledgements

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