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Simultaneous determination of *E*-2-nonenal and β-damascenone in beer by reversed-phase liquid chromatography with UV detection

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Abstract

A method for the simultaneous determination of *E*-2-nonenal and β-damascenone in beer by reversed-phase liquid chromatography using UV detection is presented. The method consists of beer steam distillation, followed by an extraction/concentration step using Sep-Pak Plus C_{18} RP cartridges and determination by HPLC at 226 nm UV-absorption maximum. The identity of the compounds was confirmed by GC analysis with MS detection of the isolated fractions. A recovery factor of approximately 80% was obtained for β-damascenone with a R.S.D. of 3%. *E*-2-Nonenal and β-damascenone were monitored in a comparative study of fresh and either naturally and forced aged beer. The results obtained show that both compounds have a similar behaviour through an extended storage of beer and consequently can be used as good analytical markers of beer ageing. Nevertheless, the use of β-damascenone seems to be more convenient because this compound appears in beer in higher concentrations than *E*-2-nonenal, thus making it easier to measure.

Keywords: Beer; Food analysis; Nonenal; Damascenone; Carbonyl compounds

1. Introduction

Flavour stability, an important quality criterion for beer, has long been a concern to the brewing industry. During storage, beer quality is gradually decreased and the production of stale flavour, formation of haze and browning occurs. The off-flavours that typically develop in aged beer include cardboard, sweet and toffee notes [1]. Although these flavours are easily identifiable to sensory analysts trained in the assessment of beer flavour, there is a definite requirement for analytical methods that may detect the specific compounds responsible for these flavours and thereby enable quantitative determination of beer staling. However, many of these compounds are either unknown or are present at such low concentrations that their analysis is difficult and inaccurate.

Carbonyl compounds, particularly aldehydes, are considered to play an important role in the deterioration of beer flavour and aroma during storage. *E*-2-Nonenal has received particular attention as the major source of the papery/cardboard character developed in aged beers [2]. An increase of β-damascenone concentration during an artificial ageing of a variety of commercial Belgian beers has been recently reported [3]. Fickert and Schieberle [4] identified β-damascenone as an odour-active volatile in an extract from a barley malt.

β-Damascenone, a terpenic ketone first isolated from Bulgarian rose oil [5] and later found in the essential oils of other natural materials, is a highly odoriferous compound important in the creation of modern fragrances [6]. As it is characterized by a very low odour threshold in water (0.02–0.09 ng/g) [7], β-damascenone was considered to be a significant flavour in many alcoholic beverages such as whiskey, brandy, rum, wine and beer [8]. β-Damascenone has been identified as a key odour in a variety of fruits, vegetables and derived products, including wine where it imparts “stewed apple”, “fruity” and honey-like character. Recent studies have indicated that β-damascenone in wine results from the hydrolytic breakdown of complex grape-derived secondary metabolites formed from carotenoids such as neoxanthin [9,10]. Kotseridis et al. [11] shown that the levels of free-damascenone of wine almost double by heat treatment. They found a content of 5.4 μg/l for free-damascenone in red wine from Bordeaux.

A method for the determination of *E*-2-nonenal in beer by reversed-phase liquid chromatography was recently

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presented [12]. A much larger unknown chromatographic peak, correlating well with beer ageing, was found close to the *E*-2-nonenal peak. This study shows how the work developed on the isolation, concentration and identification by GC analysis with MS detection, allowed us to recognize β-damascenone as the compound responsible for that peak. The presented method is applied in a comparative study of fresh and either naturally and forced aged beer.

2. Materials and methods

2.1. Instrumentation

2.1.1. Extraction

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

2.1.2. HPLC analysis

A Gilson HPLC instrument was used, consisting of a 307 pump, a 115 UV variable wavelength detector, selected at 226 nm, a Rheodyne 7125 injection valve with a 20 or 100 μl loop and a Spectra-Physics Data Jet CH1 integrator. A precolumn Nucleosil ODS (8 mm × 4 mm, 5 μm particle size) and a column Nucleosil ODS (250 mm × 4.6 mm, 5 μm particle size) from Macherey-Nagel were used. A water–acetonitrile (45:55, v/v) mixture was used as mobile phase at a flow-rate of 1.00 ml/min. The mobile phase was filtered and degassed in a vacuum filter holder, Schleicher & Schuell GV 050/0, with 0.2 mm Schleicher & Schuell NL 16 membrane filters. A Gilson Fraction Collector, Model 201, was used for the isolation of the compounds.

2.1.3. GC–MS analysis

A Saturn II (Varian) ion trap mass spectrometer (multiplier voltage: 2550 V; emission current: 10 μA; scan rate: 0.60 s; detector temperature: 190 °C; mass range *m/z*: 30–250) was used coupled with a Varian 3400 gas chromatograph, equipped with a capillary column CP-Wax 52CB from Varian (60 m × 0.25 mm i.d., 0.50 μm film thickness). Oven temperature was programmed as follows: 40 °C for 20 min, 1.5 °C/min to 200, 200 °C for 4 min, 10 °C/min to 250, 250 °C for 120 min. The injector was programmed as follows: 120 °C for 0.1 min, 180 °C/min to 250, 250 °C for 13.9 min. The helium carrier gas flow rate was 1.00 ml/min. Qualitative GC–MS analyses employed the US National Institute of Standards and Technology (NIST) 98 Mass Spectral Library for attempting the identification of the compounds.

2.2. Chemicals

Acetonitrile (HPLC grade), ethyl acetate (pure analytical grade) and sodium chloride (analytical reagent grade) were purchased from Merck. *E*-2-Nonenal (analytical reagent grade) was obtained from Sigma–Aldrich. β-Damascenone (>95%) was kindly provided by SGP Selin, Grasse, France. The water used to prepare all solutions was deionised, distilled and further purified in a Millipore system (Simplicity 185). Stock solutions of *E*-2-nonenal and β-damascenone were prepared weekly in water–acetonitrile (50:50, v/v). In this medium, the compounds are soluble and stable.

2.3. Beers

One brand of a commercial lager beer, produced at industrial scale, was used. Bottled beers with a total O_2 content less than 0.5 mg/l under different storage conditions were tested: fresh beers (maintained at 4 °C), artificially aged beers (7 days at 37 °C in the dark) and naturally aged beers (maintained at 20°C in the dark).

2.4. Experimental procedure

E-2-Nonenal and β-damascenone were extracted from beer and quantified by the method developed by Santos et al. [12]. This method is divided in three main steps: (1) steam distillation of beer; (2) passage of the distillate through a C_{18} SPE column; (3) elution and analysis by HPLC with UV detection. Before the distillation step, the ionic strength of the beer sample (250.0 ml) was adjusted by addition of 2.9 g of sodium chloride and the formation of foam was prevented by addition of one drop of an antifoaming agent. The collected distillate (15 ml) was diluted to 100 ml with water and was passed through the SPE column. After draining the column with vacuum, the retained *E*-2-nonenal and β-damascenone were extracted with 1.00 ml of acetonitrile and 20.00 μl of this extract were injected into the HPLC column.

3. Results and discussion

3.1. Isolation and identification of β-damascenone

In a previous publication [12] it was shown that the chromatographic peak of an unknown compound found next to the peak of *E*-2-nonenal correlated well with beer ageing. As that peak is much larger than the *E*-2-nonenal peak it was mentioned that it could be much easier to use it in the analytical assessment of beer ageing. In this work studies were conducted in order to isolate and identify the compound responsible for that peak.

The isolation, concentration and identification of the unknown compound was started by a steam distillation of 250.0 ml of beer with collection of 50 ml of distillate. Before the distillation step, the ionic strength of beer was adjusted by addition of sodium chloride; one drop of antifoaming agent was added to prevent foam formation. Beer was acidified to pH 2 with concentrated hydrochloric acid, because higher quantities of *E*-2-nonenal and of the unknown

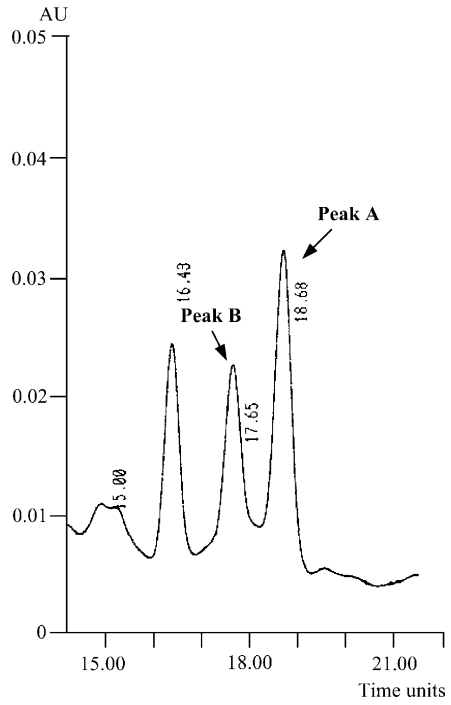

Fig. 1. Chromatogram obtained in the HPLC-UV analysis of an extract of acidified beer (pH 2). Peaks: (A) unknown compound; (B) *E*-2-nonenal.

compound are produced when the distillation occurs in more acidic conditions [12]. The distillate was diluted with water to 100 ml and was passed through a C_{18} SPE column. After draining the column with vacuum, the retained compounds were removed with 500 μl of acetonitrile (concentrated extract). Five injections of 100 μl of this extract were made into the HPLC column and the compound responsible for the unknown peak was isolated using a fraction collector. Fig. 1 illustrates a chromatogram obtained in the analysis of a concentrated extract where peaks A and B correspond, respectively to the unknown compound and to *E*-2-nonenal.

The fractions collected in the five injections were mixed, diluted with water to 100 ml and passed through another C_{18} SPE column. The dilution with water was necessary because the undiluted mix had a high content in acetonitrile, which would hinder the retention of the unknown compound in the SPE column. The column was drained with vacuum and the retained compounds were removed with 250 μl of ethyl acetate (final extract A). A 2 μl aliquot of this extract was injected into the GC column and the compounds present were detected by MS. The whole process was also applied to the peak assigned as *E*-2-nonenal in order to confirm its identity (final extract B). GC–MS chromatograms obtained for the final extracts A and B are illustrated in Fig. 2. Although the retention times of the studied compounds were not known in these conditions, a detailed comparison of the chromatograms enabled us to recognize the specific chromatographic signals of each of the compounds (indicated by arrows in Fig. 2). Based on mass spectrum and kovats index, it was possible to identify compound A as β-damascenone; in a similar way, it was also confirmed that compound B is *E*-2-nonenal (data not shown).

3.2. Assay for the evaluation of beer ageing

3.2.1. Determination of β-damascenone in beer

In order to obtain an accurate quantification of β-damascenone in beer it is necessary to take into consideration any losses of the compound that can occur during the extraction procedures. As it can be seen in Table 1, only about 80% of β-damascenone added to beer samples was recovered at the end of the extraction. In order to avoid this problem, β-damascenone quantification was made using the method of standard additions, with the standards being added to the beer before the distillation.

An example of the application of the method in the determination of β-damascenone in an aged beer (4 weeks of storage at 20 °C) is illustrated in Fig. 3. As it can be seen

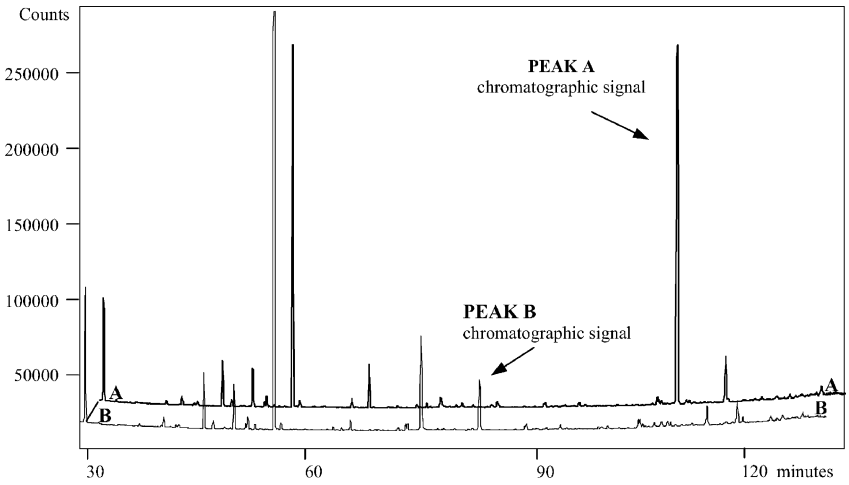

Fig. 2. GC–MS chromatograms obtained for: final extract A, on the top; final extract B, on the bottom (chromatogram A is slightly deviated to allow a better view of both chromatograms). The specific chromatographic signals which allowed the identification of the compounds are indicated by the arrows.

Table 1
Recovery of β-damascenone in the extraction/concentration process

Assay	Beer spiked with β-damascenone			Direct injection of β-damascenone			Recovery ($\Delta A/\Delta B$) (%)
	β-Damascenone (10^{-8} mol/l)	Peak area	Variation of peak area (ΔA)	β-Damascenone[a] (10^{-6} mol/l)	Peak area	Variation of peak area (ΔB)	
1	x (beer)	13 490	–	0	0	–	–
2	$x + 0.80$	20 951	7 461	2.00	10 176	10 176	73
3	$x + 1.60$	28 527	15 037	4.00	18 846	18 846	80
4	$x + 2.40$	36 312	22 822	6.00	28 014	28 014	82
5	$x + 3.20$	45 684	32 194	8.00	36 372	36 372	88
6	$x + 4.00$	51 678	38 188	10.00	45 696	45 696	84

[a] 250 times more concentrated, as there is a 250-fold concentration during extraction.

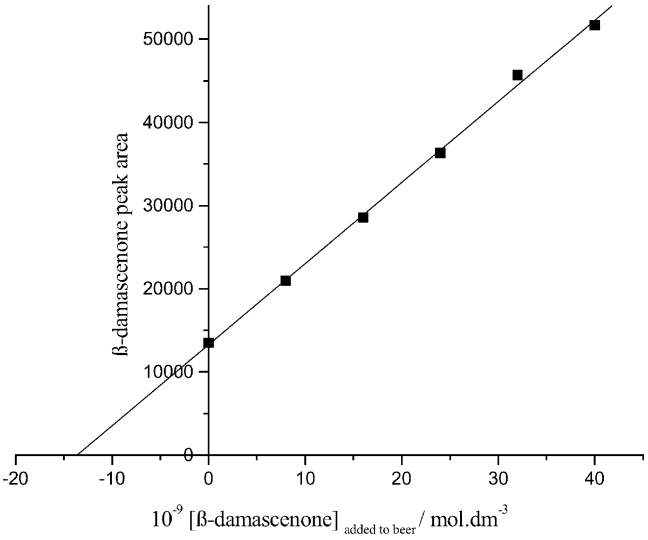

Fig. 3. Determination of β-damascenone in a beer stored during 4 weeks at 20 °C using the method of standard additions (standards added before extraction).

in Table 2, a R.S.D. lower than 3% was obtained in the determination of β-damascenone in ten replicates of a beer sample, showing that the method has a good repeatability.

3.2.2. Effect of storage in the concentration of E-2-nonenal and β-damascenone found in beer

Beer flavour deterioration at high temperatures is a well known problem that brewers must face-up to. *E*-2-Nonenal and β-damascenone were monitored throughout an extended storage of beer in order to try to obtain a correlation between the storage conditions and the concentrations of the compounds. Two different lots of beer were stored in the following conditions: 4 weeks at 4 °C, 4 and 12 weeks at 20 °C in the dark (naturally aged beers) and 7 days at 37 °C in the dark (artificially aged beers). A duplicate of beers were analyzed for each condition.

Table 3
Effect of the time and of the temperature of storage on the concentration of *E*-2-nonenal and β-damascenone found in beer

Lot	Fresh beer	4 °C (12 weeks)	20 °C (4 weeks)	20 °C (12 weeks)	37 °C (1 week)
E-2-Nonenal (μg/l)					
A	0.06	0.07	0.08	0.13	0.16
B	0.07	0.07	0.09	0.18	0.20
β-Damascenone (μg/l)					
A	1.8	1.6	2.3	2.6	2.9
B	1.8	1.8	2.2	2.6	2.8

As it can be seen in Table 3, there is no variation on the concentrations of *E*-2-nonenal and β-damascenone for beers stored at 4 °C, although the concentration of both compounds increases for beers stored at higher temperatures. It is worthy to note that the concentration of *E*-2-nonenal after 3 months of natural ageing already exceeds its flavour threshold in beer (roughly 0.1 μg/l).

High storage temperatures led to the formation/liberation of β-damascenone, resulting in an increase of the concentration of this compound during beer ageing. Although the mechanisms responsible for this behaviour have not been completely elucidated yet, the acidic hydrolysis of glycosides has been described in literature as the most likely hypothesis [3,10].

In order to evaluate the effect of beer pH in the concentration of β-damascenone, several beers were analyzed after a pH adjustment. The results obtained (Fig. 4) confirm that the formation/liberation of β-damascenone from its precursors is higher for lower pH values, thus supporting the

Table 2
Determination of β-damascenone in a beer sample using the proposed method of analysis

	Replicates									
	1	2	3	4	5	6	7	8	9	10
β-Damascenone (10^{-9} mol/l)	8.4	8.9	8.6	9.2	9.0	8.9	8.6	9.0	9.0	8.7

Average concentration, 8.83×10^{-9} mol/l; standard deviation, 0.24×10^{-9} mol/l; R.S.D., 2.7%.

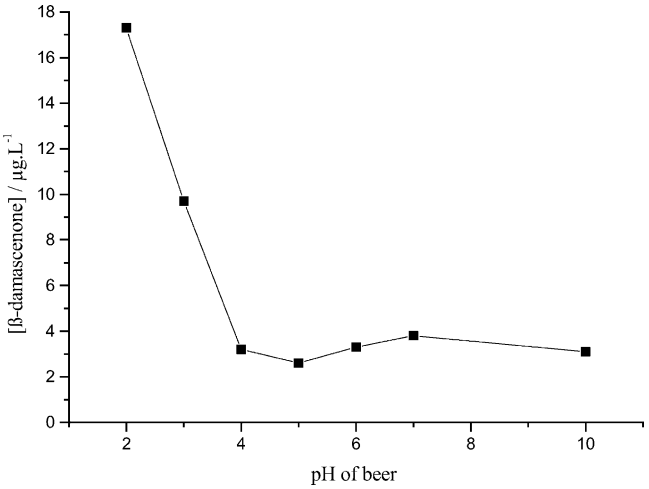

Fig. 4. Effect of pH on the concentration of β-damascenone found in beer using the proposed method of analysis.

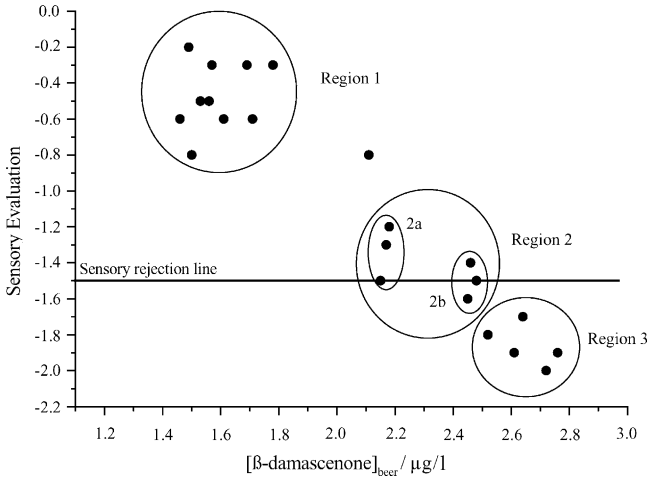

Fig. 5. Relationship between sensory evaluation and β-damascenone concentration found in beer. Region (1): fresh beer and beer stored at 4 °C during 4 or 12 weeks; region (2a): beer stored 4 weeks at 20 °C; region (2b): beer stored 12 weeks at 20 °C; region (3): beer stored 1 week at 37 °C.

hypothesis that an acidic hydrolysis is responsible for the development of β-damascenone in beer. These results are in agreement with those obtained by Gijs et al. [13].

3.2.3. β-Damascenone as an analytical marker of beer ageing

A more specific study was performed to evaluate the possible correlation between the β-damascenone content and the development of off-flavours during beer ageing, with the aid of a sensory panel. β-Damascenone was determined in several beers stored for a long period of time in different conditions and, simultaneously, the sensory changes were followed regularly by an expert evaluation panel. This panel consisted of eight trained tasters who were asked to comment on general quality (discrimination test) as well as to describe the flavour profile according to a special form (description test). The beers were tasted by the Duo–Trio test, in which the same beer stored at 4 °C during the same time was the reference. All the beers were tasted at 4 °C and evaluated according to the following scale: (+1) without any defects normally found in this kind of product; (0) normal for this kind of product; (−1) with acceptable defects for this kind of product; (−2) with non acceptable defects for this kind of product; (−3) with defects so serious that an immediate action is required. The sensory classification of each beer consisted in the mean value of the classifications provided by all the tasters, with a sensory rejection limit of −1.5 being adopted. The organoleptic state of a beer with a classification lower than −1.5 is considered not satisfactory, implying the rejection of the product by the panel.

As it can be seen in Fig. 5, a good correlation was obtained between the concentration of β-damascenone in beer and the sensory evaluation, with higher concentrations of β-damascenone corresponding to lower values of the sensory evaluation. A more detailed inspection of Fig. 5 allows the identification of three different regions. Region 1 includes beers analyzed immediately after bottling and beers stored at 4 °C during 4 and 12 weeks; these beers obtained the highest classifications in the sensory evaluation tests and contained the lower concentrations of β-damascenone. Region 2 is formed by beers stored at 20 °C; beers stored during 12 weeks (sub-region 2b) had a slightly worse sensory evaluation than beers stored during 4 weeks only (sub-region 2a), approaching the sensory rejection line; this decrease in the sensory evaluation was accompanied by an increase in the concentrations of β-damascenone. Finally, all the beers of region 3 (stored at 37 °C during 1 week) were considered not satisfactory by the sensory panel, and this worse evaluation was associated to a high concentration of β-damascenone.

The observed correlation between the β-damascenone content and the storage temperature of beer shows that this can be used as a good analytical marker of beer ageing induced by heat. Additionally, the obtained results suggest that β-damascenone can also be used as an analytical marker of the organoleptic state of beer. Obviously, this does not imply that β-damascenone has a direct impact on the organoleptic degradation that beer suffers during its ageing, or that the off-flavours perceived by the sensory panel are consequence of concentration of β-damascenone present.

4. Conclusions

A new method for the determination of β-damascenone, an indicator of beer ageing, is presented. Although the investigation of the mechanisms of formation of β-damascenone during beer ageing is just beginning, this compound can be

very useful as a good analytical marker of heat damage of beer. Additionally, it was shown that the β-damascenone content of beer correlates well with the total taste score attributed by an expert sensory panel. Work is in progress in order to study the effect of raw materials on the formation/liberation of β-damascenone throughout the brewing process. At present, a relationship between the increase of β-damascenone and *E*-2-nonenal during beer ageing cannot be excluded.

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