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Note

High-performance liquid chromatography of the bis(dinitrophenyl)hydrazone derivative of ascorbic acid, applied to analysis of ascorbic acid in beer

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Ascorbic acid is important not only because of its vitamin C activity but also because of its use as a food additive (mainly as an antioxidant). In the latter case, characteristic degradation products of ascorbic acid, namely dehydroascorbic acid and diketogulonic acid, may also be present. Ascorbic acid itself is readily determined by high-performance liquid chromatography (HPLC) with ultraviolet detection, *e.g.*, ref. 1, but methods to determine dehydroascorbic acid and diketogulonic acid by HPLC with ultraviolet detection or refractive index detection are too insensitive[2–4] for practical applications in foodstuffs. Therefore a derivatization procedure is needed. Precolumn derivatization[5] and postcolumn derivatization[6] methods have been described in which ascorbic and dehydroascorbic acid could be determined, but not diketogulonic acid.

It is possible to derivatize diketogulonic acid with 2,4-dinitrophenylhydrazine (DNPH), known as the Roe–Kuether method[7]. After transformation of ascorbic acid and dehydroascorbic acid into diketogulonic acid by oxidation and reaction of the latter with DNPH, the bis(dinitrophenyl)hydrazone derivative of ascorbic acid is formed and determined by spectrophotometry. The derivatization is, however, not specific since certain components in foodstuffs such as sugars and reductones may react with DNPH, the more so at elevated temperatures; therefore the reaction is usually carried out at, for instance, 20°C for 16 h[8]. Paper chromatography[9], thin-layer chromatography[8,10] and column chromatography[11] have been used to separate the bishydrazone derivative of ascorbic acid from interfering hydrazones, but these methods are rather tedious.

HPLC has also been used[12] for the analysis of the bishydrazone derivative of ascorbic acid in a relatively simple system (aqueous humor from cataract patients). However, the separating power of HPLC offers the possibility to analyse more complex reaction mixtures from the reaction of DNPH with foods containing ascorbic acid, so that the reaction may be accelerated by applying a high temperature.

In this note we describe a relatively fast method to determine the sum of ascorbic acid, dehydroascorbic acid and diketogulonic acid in foodstuffs by HPLC

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analysis of the bishydrazone derivative of ascorbic acid. Beer was chosen as a model system since it is known to contain many components (such as reductones) that react with DNPH[13].

EXPERIMENTAL

Reagents

2,4-Dinitrophenylhydrazine, ascorbic acid, thiourea, acetic acid, sodium acetate, sulphuric acid, iodine and ethylacetate were obtained from E. Merck (Darmstadt, G.F.R.). Acetonitrile was obtained from Baker Chemicals. Distilled water was used to prepare solutions.

Three grams of 2,4-dinitrophenylhydrazine (DNPH) were suspended in 50 ml glacial acetic acid and dissolved by adding 50 ml of concentrated sulphuric acid[10].

Derivatization

To 15 ml beer or standard solution of ascorbic acid were added six to ten drops of a 0.1 *N* I_2 solution until a brown colour persisted. The mixture was shaken and after 5 min a few crystals of thiourea were added until the brown colour disappeared. Then 4 ml of the DNPH solution were added; the solution was mixed and kept at 37°C for 16 h or 70°C for 2 h. Afterwards, 20 ml water were added and the solution was cooled to 5°C for 15–20 min. The red crystals formed were filtered over sintered glass (Schott Jena G 3) and washed with 60–80 ml water. They were dissolved in 50 ml ethyl acetate and 15 μl of this solution were injected onto the HPLC column.

Apparatus

A Waters HPLC pump, Model 6000A, a Waters autosampler (WISP 710 A) and a Waters recorder/integrator (Data Module) were used. A variable-wavelength detector equipped with a mode for wavelength scanning was used (Perkin-Elmer LC-75 with autocontrol).

Chromatographic conditions

The mobile phase was 50% acetonitrile in a 0.01 *M* sodium acetate buffer (pH 4.1). The eluent was filtered through a 0.22-μm Millipore filter and subsequently degassed by applying a vacuum. The flow-rate was 2 ml/min. HPLC was performed at ambient temperature.

The column (250 × 4.6 mm I.D.) was packed with LiChrosorb 10 RP-18, particle size 10 μm (Chrompack, The Netherlands).

The absorption of the eluent was recorded at 497 nm.

RESULTS AND DISCUSSION

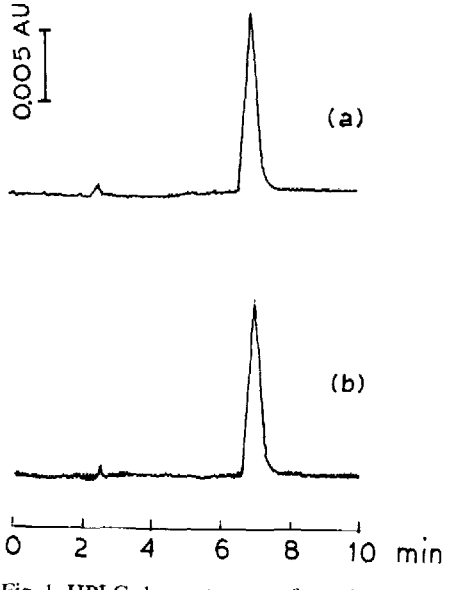
The reaction products formed after incubation with DNPH may be obtained by extraction with ethyl acetate, *e.g.*, ref. 10, or by filtration of the crystals formed after cooling and subsequent dissolution in ethyl acetate[11]. The filtration method is recommended since extraction is hampered by formation of an emulsion. Moreover,

filtration and subsequent washing of the crystals leads to a lower number of peaks on the chromatogram.

The DNPH reaction is not specific for ascorbic acid degradation products and the higher the temperature the more reaction products other than the bishydrazone derivative of ascorbic acid are formed. For this reason, a reaction temperature of 20°C was recommended for the reaction of DNPH with beer for 16 h[8]. We increased the reaction temperature to 37°C and kept the reaction time at 16 h, but a lager beer with ascorbic acid showed only one peak on HPLC analysis (Fig. 1) and a lager beer without ascorbic acid showed no peak at all. The reaction products were detected at 497 nm since this was found to be the absorption maximum of the bishydrazone derivative of ascorbic acid in this particular eluent (as determined from a spectrum of the peak). It is, however, possible to use 254 nm also, but then peaks of unreacted DNPH and impurities in ethyl acetate are also detected[12].

A further increase of the reaction temperature decreases the necessary reaction time considerably. Fig. 2 shows the reaction products obtained after incubation at 70°C for 2 h. It is seen that more reaction products are formed but these are separated from the ascorbic acid derivative. The number of reaction products depends, of course, on the particular sample. We investigated 27 samples of different kinds of beer and no interfering peaks were found, although baseline separation was not always obtained. Fig. 3 shows a complex chromatogram of the products of reaction of DNPH with Gueuze Lambic (Belgian beer) with and without ascorbic acid added.

The recovery of ascorbic acid added to lager beer was 100 ± 2% (n = 5)



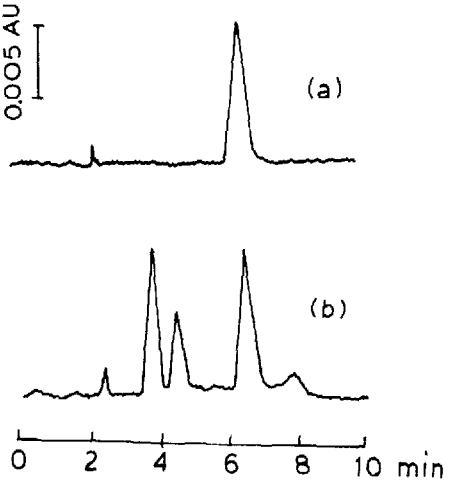


Fig. 1. HPLC chromatogram of reaction products formed at 37°C after 16 h. (a) 25 mg/l ascorbic acid in water; (b) 25 mg/l ascorbic acid in lager beer. Detection is at 497 nm.

Fig. 2. HPLC chromatogram of reaction products formed at 70°C after 2 h. (a) 20 mg/l ascorbic acid in water; (b) 20 mg/l ascorbic acid in lager beer. Detection is at 497 nm.

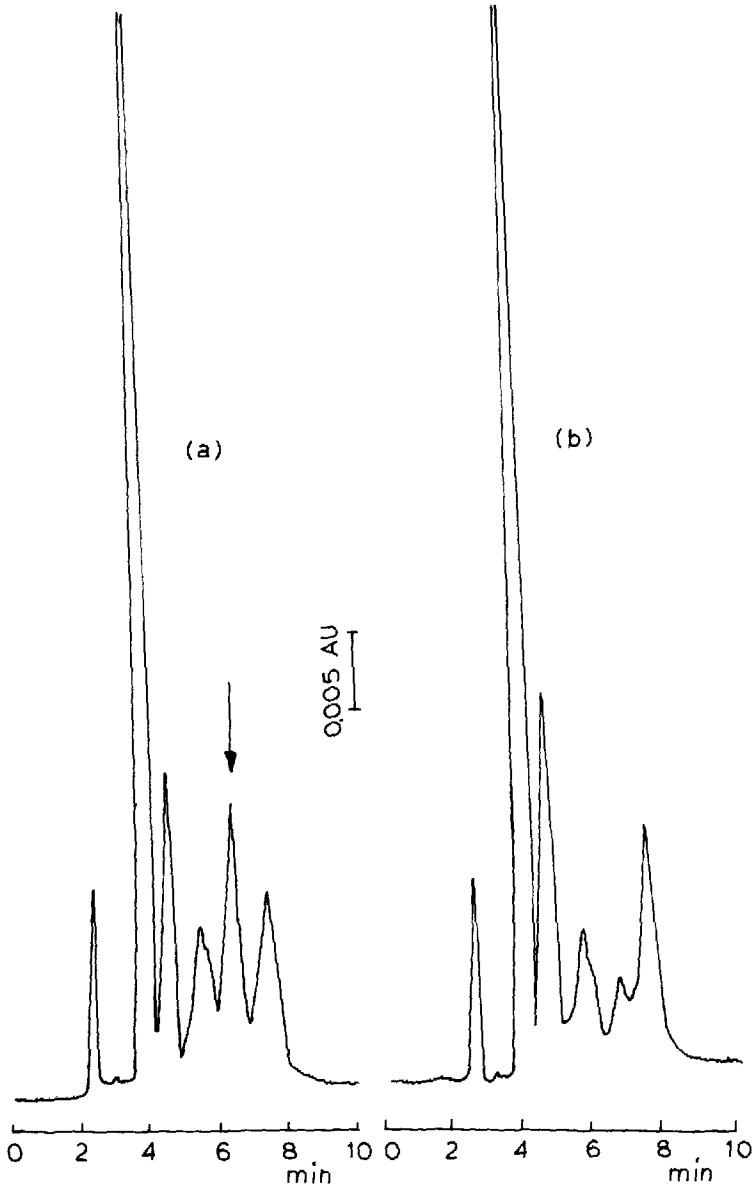


Fig. 3. HPLC chromatogram of reaction products formed at 70°C after 2 h. (a) 25 mg/l ascorbic acid in Gueuze Lambic, the arrow indicating the bishydrazone derivative of ascorbic acid; (b) Gueuze Lambic without ascorbic acid. Detection is at 497 nm.

when the reaction was carried out at 70°C for 2 h. The limit of detection is about 2 mg/l ascorbic acid.

This method may be applicable to other food products and perhaps even higher temperatures may be used, so that the reaction time can be further reduced. This eliminates a major drawback of the DNPH reaction in the analysis of ascorbic acid and its degradation products in foodstuffs.

REFERENCES

1 J. Geigert, D. S. Hirano and S. L. Neidleman, *J. Chromatogr.*, 206 (1981) 396.
2 L. W. Doner and K. B. Hicks, *Anal. Biochem.*, 115 (1981) 225.
3 J. W. Finley and E. Duang, *J. Chromatogr.*, 207 (1981) 449.
4 R. C. Rose and D. L. Nahrwold, *Anal. Biochem.*, 114 (1981) 140.
5 R. W. Keating and P. R. Haddad, *J. Chromatogr.*, 245 (1982) 249.
6 R. L. Veazey and T. A. Nieman, *J. Chromatogr.*, 200 (1980) 153.
7 J. H. Roe and C. A. Kuether, *J. Biol. Chem.*, 147 (1943) 399.
8 W. Postel, *Brauwiss.*, 25 (1972) 355.
9 K. Szöke Szotyori, *Nahrung*, 11 (1967) 421.
10 H. Thaler and U. Gieger, *Mitt. Lebensm. Unters. Hyg.*, 58 (1967) 473.
11 L. W. Mapson, *Biochem. J.*, 80 (1961) 459.
12 S. Garcia-Castiñeiras, V. Díaz Bonnet, R. Figueroa and M. Miranda, *J. Liquid Chromatogr.*, 4 (1981) 1619.
13 J. Jerumanis, G. Rieder and A. Devreux, *Brauwiss.*, 28 (1975) 19.