Solid-state $^{13}$C NMR investigations of insoluble deposits in aromatic bitters

Abstract Insoluble deposit formed in aromatic bitters produced from alcoholic extracts of about 30 different botanical materials with a dry matter content of 1.67% and 38 vol% alcohol and a characteristic astringent flavour was shown by $^{13}$C NMR spectroscopy to be a coprecipitate of plant polyphenols, proteins and carbohydrates. The polyphenols in the deposit were flavonoids such as anthocyanidins and anthocyanins, with glucose as the principal carbohydrate and derivatives of cinnamic acid. The deposit formation in the aromatic bitters was accelerated by addition of the radical initiator 2,2'-azobis(2-amidinopropane) dihydrochloride or by addition of hydrogen peroxide in combination with iron(III) nitrate and ascorbic acid as a Fenton reagent. Polymerization of catechin and pelargonidin was shown, in model experiments, to be oxidative and to be accelerated by exposure to light. A mechanism involving oxidative polymerization of polyphenols followed by coprecipitation of polymerized polyphenols, proteins and carbohydrates is proposed for formation of deposits in alcoholic beverages of the bitters type.

Key words Bitters · Deposit · Polyphenol polymerization · Solid-state $^{13}$C NMR spectroscopy

Introduction

Alcoholic beverages with extracts of different botanical materials have a long tradition in several European regions and a bitter taste and a marked astringency are essential for the flavour of these products. Due to the high content of dry matter, several of the bitters have stability problems upon exposure to light or as a result of temperature fluctuations or of prolonged contact with air. Formation of turbidity and deposit are characteristic of a number of beverages of plant origin and often present problems for marketing. Haze formation in beer has thus been shown to depend on acid-catalysed degradation of proanthocyanidins with intermediate generation of flavan-3-ol-based carbocations [1], which subsequently are captured by nucleophilic groups on proteins. This results in complex formation between proanthocyanidins and proteins [1,2]. The haze formed in beer is thus composed of three different structural elements, i.e. phenolic compounds, protein and carbohydrate [1], and the deposit formed in red wine has a similar composition [3]. The mechanisms behind the polyphenolic polymerization in red wine is, however, still uncertain, although the polymerization is believed to be initiated by an acid-catalysed condensation of procyanidins and anthocyanins with some contribution from the Baeyer reaction in which $-\text{CH(CH}_3)\text{-}$ bridges between phloroglucinol rings are formed from acetaldehyde [4-6].

Wine deposit formation does not effect the flavour [3], but it has been recognized that certain bitters get more sweet and less astringent when they turn turbid compared to the freshly bottled product [7]. The loss of astringency is thought to be the result of polymerization and precipitation of astringent flavonoids, such as procyanidins [4, 8, 9]. Beer brewed with proanthocyanidin-free malt has a similar astringency as beer brewed with regular malt which, however, is more bitter and more easily forms haze during storage [10]. Similar knowledge is not available for aromatic bitters, and we have undertaken a solid-state $^{13}$C NMR study of deposit formed in this
type of alcoholic beverage. The investigations further provide the basis for a discussion of the mechanisms of the formation of deposit.

**Materials and methods**

**Materials.** Test bitters (38 vol% molasse spirit) were produced by Danisco Distillers (Copenhagen, Denmark) and contained distillates and extracts of about 30 different seeds, drugs and herbs. D-Galacturonic acid monohydrate, D(+)-galactose, (-)-arabitol, caffeic acid, ferulic acid and quercetin were from Sigma (St. Louis, Mo., USA). D(+)-glucose monohydrate, iron(III) nitrate nonahydrate, acetaldehyde and L-(+)-ascorbic acid were from Merck (Darmstadt, Germany). (+)-Catechin, pelargonidin chloride and p-coumaric acid were from Fluka (Buchs, Switzerland). (−)-Cyanidin, pelargonidin chloride, which were added to 40 mmol/l AAPH, or 0.14 mol/l acetaldehyde were added, and the mixture was saturated with oxygen by bubbling with oxygen for 30 min. The bitters were stored at 25°C and analysed for high molecular products by thin-layer chromatography (TLC) as described by Asano et al. [2]. Before the TLC analyses, 1 ml sample solution was evaporated to dryness by vacuum centrifugation and the resulting material was dissolved in 0.1 ml ethanol. Aliquots of 10 μl were spotted onto a silica gel 60 plate (Merck) and developed with toluene/acetone/formic acid (4:6:1 v/v/v). The plate was then sprayed with vanillin-HCI reagent (10% vanillin in ethan-1ol-37 vol% HCl, 5:3 v/v). For elution, acetonitrile/water (4:1 v/v) with a flow rate of 0.30 ml/min was used, and the column temperature was 30°C. Samples of 10 μl were injected and a Milton Roy refractometer monitored IV (Riviera Beach, Fla., USA) as detector.

**Elemental analysis.** Elemental analysis was performed for hydrogen, carbon, nitrogen and sulphur on a Perkin Elmer 2400 CN elemental analyser (Norwalk, Conn., USA) at the Microanalytical Laboratory of the H.C. Orsted Institute, University of Copenhagen.

**Sugar analyses.** Uronic acid content in the deposit was determined, after hydrolysis with 2 mol/l trifluoroacetic acid (120°C, 75 min), by gas chromatography of trimethyl derivatives essentially as described by Waters et al. [3]. The trimethylsilyl derivatives were prepared by derivatization of the dry hydrolysate with 200 μl Tri-Sili TBT reagent from Pierce (Rockford, Ill., USA) at 60°C in 30 min. Ultra 2 column (25 m × 0.20 mm, 0.33 μm; Hewlett-Packard) with a flow rate of 0.6 ml/min, split injection with injection temperature 270°C, flame ionization detector 280°C. The temperature programme was: 130°C in 3 min then 4°C/min for 23 min.

**Amino acid analysis.** The deposit was hydrolysed with 6 mol/l HCl for 12 h at 110°C. The amino acid analyses were performed according to Bidlingmeyer et al. [12] on a Waters PICOTAG system (Milford, Mass., USA).

**Polyphenol analyses.** Cyanidin has been shown to be formed by acid hydrolysis of proanthocyanidins [13], and the detection of anthocyanidins after hydrolysis in methanol: HCl (6 mol/l) (1:1 v/v, 100°C, 30 min) gave an indication of the presence of proanthocyanidins in the deposit. Anthocyanidins and other polyphenols were detected in the hydrolysate after separation by an HPLC method modified from Castelde et al. [14] using an ODS-Hypersil column (250 x 4.6 mm, 5 μm; Hewlett-Packard) and eluting with a gradient of 5% formic acid (solvent A) / methanol (solvent B) 1 ml/min at 35°C with on-line diode array detection (Hewlett-Packard). The gradient used was: 7% B in 2 min, 7–15% B in 12 min, 15–75% B in 34 min, 75–80% B in 4 min, 80% B in 4 min, 80–100% B in 10 min, 100% B in 4 min and 100–7% B in 10 min. Samples of 20 μl were injected.

**Results**

**Deposit composition**

**Solid-state 13C NMR spectroscopy of the deposit from the aromatic bitters gave a spectrum (Fig. 1A) that showed well-defined signals for at least three different structural categories which were identified as proteins, polyphenols and carbohydrates.**

The sharp signal at 52.6 ppm is assigned to OCH3 groups of proanthocyanidins and other aromatic systems. The signal at 104 ppm was due to flavonoid material, preferentially quaternary carbons such as C4a