Components Contributing to Beef Flavor

Isolation of 2-Acetyl-2-thiazoline from Beef Broth

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A flavor concentrate prepared by continuous extraction with diethyl ether of a clear broth was subjected to gas–liquid chromatography. A compound with an intense aroma of freshly baked bread crusts was detected in the effluent. This trace constituent was isolated and purified by the following procedure. A broth prepared from 225 kg of lean shin of beef was filtered and continuously extracted with diethyl ether. The ether was evaporated from the extract and gradually replaced by distilled water. The aqueous phase was saturated with sodium bicarbonate and then continuously extracted with pentane. The pentane extract was concentrated and purified by liquid–liquid chromatography. The fraction containing the aroma component was further purified by repeated gas–liquid chromatography over two different stationary phases. The pure compound was finally trapped and identified as 2-acetyl-2-thiazoline on the basis of its infrared and mass spectra.

Although many compounds contributing to the flavor of cooked beef have been identified during the last decade [see for example the lists published by Herz (1968) and Tonsbeek *et al.* (1968)], one can be sure that there are as yet unidentified trace materials which contribute significantly to the overall flavor of cooked beef. This paper describes the isolation and identification of such a trace compound.

During the course of our work on the flavor components of beef broth (Tonsbeek *et al.*, 1968) we subjected a concentrated ether extract of beef broth to gas–liquid chromatography and continuously monitored the odor of the eluting materials. A compound with an intense odor of freshly baked bread crust was eluted between isocaproic acid and caproic acid in quantities insufficient to elicit a response from the flame ionization detector. Injection of larger volumes of concentrate resulted in an enhancement of the observed odor but, of course, also resulted in increased quantities of the caproic acids. Since these two acids were present in large quantities relative to the unknown, it was obvious that in order to isolate a sufficient amount of the material to permit its identification we would have to use methods other than glc to separate the fatty acids from the odorous substance, and we would have to extract large quantities of beef.

The concentrated ether extract obtained from beef broth was freed of fatty acids by transferring the flavor components from the ether solution to aqueous solution, saturating the latter with $NaHCO_3$, and then continuously extracting with pentane. The volume of concentrate which could be injected onto the glc column was now found to be limited by the presence of fats. Small-scale experiments showed that the fat could be removed from the pentane extract by partition chromatography over a silicic acid/water column. An attempt to subject the complete ether extract prepared from beef broth to this type of fractionation failed because the fatty acids, especially the caproic acids, were eluted together with the flavor compound. Direct extraction with pentane of a beef broth saturated with $NaHCO_3$ proved to be impossible due to the formation of an emulsion. For these reasons the ether extraction, the pentane extraction, and the fractionation by partition chromatography all had to be applied. Once we had completed the experiments outlined above and decided upon our working-up and purification procedures, we moved on to the large-scale preparation of flavor concentrates from beef broth.

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EXPERIMENTAL

Large-Scale Preparation of Beef Broth. Sliced lean shin of beef (75 kg) was diced immediately after delivery (J-Dicer, Urschel Laboratories Inc., Valparaiso, Ind.) to cubes with sides of approximately 0.6 cm. The beef was then mixed with 75 l. of water in a double-walled, stainless steel jamboiling kettle of 200-l. capacity. The mixture was heated to 98° C by direct injection of pure steam and by passing steam between the walls of the vessel. The broth was simmered at 98° C for 2.5 hr with occasional stirring; the vessel was covered with a stainless steel lid. The broth was cooled to room temperature and the coarse pieces of beef and solidified fat were removed by sieving the broth through a stainless steel gauze (openings 0.4 × 0.4 cm). The slurry was then filtered through a Filtrox A3 paper using a filter press (Filtrox Schichtenfilter; 40 × 40 cm, Inox; Filtrox Werk A.G., St. Gallen, Switzerland). Losses by adsorption on the meat pieces and losses in the filter press reduced the yield of clear beef broth to approximately 80 l.

Preparation of an Ether Extract. The clear broth was divided into two equal portions which were each subjected to continuous extraction for 36 hr with 10-l. aliquots of predistilled diethyl ether. Due to the large diameter (22.5 cm) of the extraction vessels the solvent droplets had to be spread evenly over the extraction column by percolating the solvent through a sintered glass disk (porosity of 1). The volume of the combined ether extracts was reduced to approximately 1 l. by evaporation under reduced pressure (25 cm) in a Büchi Rotavapor. The remaining ether was evaporated as 500 ml. of distilled water was gradually added. The compound under investigation was retained in the aqueous phase during this treatment.

Preparation of a Pentane Extract Free of Fatty Acids. The aqueous solution was carefully neutralized with $NaHCO_3$ and finally saturated with this reagent. The solution was then continuously extracted for 48 hr with approximately 250 ml of pentane which had been purified by distillation over a Normag spinning-band column. The pentane extract was dried over anhydrous Na_2SO_4, and carefully concentrated to a volume of 10 ml by evaporation of the solvent using a spinning-band distillation column. This solution was shown by glc to be virtually free of fatty acids.

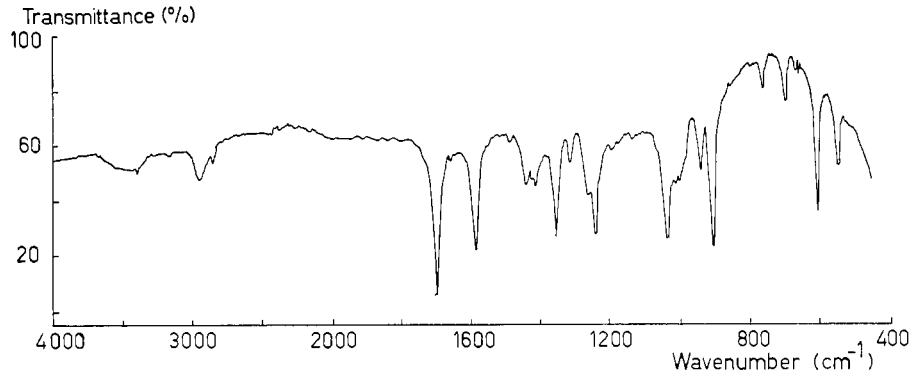


Figure 1. Infrared spectrum of 2-acetyl-2-thiazoline (in KBr disk)

Removal of Fats by Partition Chromatography. The stationary phase for the liquid–liquid chromatographic removal of fats was prepared by triturating 60 g of silicic acid (Kieselgel N HR; ex Machery, Nagel & Co.) with 34 ml of distilled water. The free-flowing powder was suspended in purified pentane. Ten water-cooled chromatographic columns (internal diameter 1.0 cm) were filled to a height of 15.0 cm with the stationary phase. The columns were then washed with pentane to remove impurities. To each of the columns was added 1 ml of the flavor concentrate. Elution of the columns was performed with pentane. With this system four fractions, each with a distinctive odor, could be obtained

Fraction	Odor
I, majority of fats	Pungent
II, remainder of fats	2-Aminoacetophenone
III, ?	Freshly baked bread crust
IV, ?	Fruity

The first 30 ml of the eluate were discarded because they contained the majority of the fats; the next 40 ml contained the flavor compound in which we were interested. The corresponding fractions from all ten columns were combined and then concentrated to 1 ml by evaporating the solvent over a spinning-band column. This solution was subjected to renewed chromatography as described above, fractions with a volume of 5 ml being collected. The fat-containing fractions were again discarded. The remaining fractions (7–13) were later combined with two more 35-ml portions containing the flavor compound. These portions were prepared in the same way from another two 75-kg batches of beef. The resulting 105 ml of pentane solution was then concentrated to 1 ml by evaporating the solvent over a spinning-band column. The remaining solution was further concentrated to approximately 250 μl by warming the vessel by hand.

Isolation of Pure Flavor Compound by Gas–Liquid Chromatography. The gas chromatograph used throughout this study was a Hewlett-Packard F&M Model 402 which had been modified as described by Van de Weerdhof *et al.* (1970) for the F&M Model 5750. The equipment was operated under the following conditions.

Gas flow rates:	Nitrogen (carrier), 45 ml/min
	Hydrogen, 20 ml/min
	Air, 100 ml/min
Temperatures:	Oven, 50–240° C; program rate 3° C/min
	F.I.D., 320° C
	Injection port, oven temperature
	Exhaust, 240° C

The split ratio exhaust/detector was 85/15 during these experiments.

A 100–125-μl sample of the pentane solution, prepared as described above, was injected on a 1-m column of internal diameter 0.3 cm packed with 20% Carbowax 20M on 60/80 mesh Diatoport S. As it was eluted, the flavor material was condensed in a cooled trap filled with 60/80 mesh Diatoport S coated with 10% Apiezon L and 1% Carbowax 20M. A second sample of 100–125 μl was chromatographed over the same column and the peak material was condensed in the same trap. The trap was inserted in front of a second glc column (length 3 m, internal diameter 0.3 cm) packed with 10% Apiezon L and 1% Carbowax 20M on 60/80 mesh Diatoport S. The peak material collected from the first column was now separated into two main components and several minor impurities. The flavor compound was condensed in a cooled trap filled with purified 60/80 mesh Diatoport S. This trapped material was used for structure elucidation by spectrometric methods. The flavor compound was transferred from the trap onto potassium bromide powder by the vacuum-deposition technique described by Copier (1968). A micro-pellet (diameter 1.5 mm) was prepared in the normal way, and the infrared spectrum (Figure 1) was recorded on a Perkin-Elmer Model 225 spectrophotometer. (By comparison of this spectrum with that of a synthesized sample of the flavor material, we later deduced that we had isolated approximately 15 μg of compound from 225 kg of beef.) The flavor compound was recovered from the pellet by extraction with diethyl ether. An attempt to measure the ultraviolet absorption spectrum of this solution failed because the concentration was too low. After concentration of the ether solution and renewed glc purification over an Apiezon L/Carbowax 20M column, the flavor compound was trapped once more on solid support. The trap was now connected to the ionization source of an AEI MS-9 mass spectrometer, and the spectrum was recorded (Figure 2).

To permit accurate measurement of the mass of the parent ion, the whole of the purified material obtained from an additional 75-kg batch of beef was required.

RESULTS AND DISCUSSION

The molecular weight of the flavor compound was found by high resolution mass spectrometry to be 129.0249, corresponding to the molecular formula C_5H_7NOS (calculated mass 129.0248). Comparison with the general formula for saturated noncyclic compounds, $C_nH_{2n+3}NOS$, shows that the flavor compound contains three double bonds and/or rings. In view of the high relative intensity of the parent peak in the

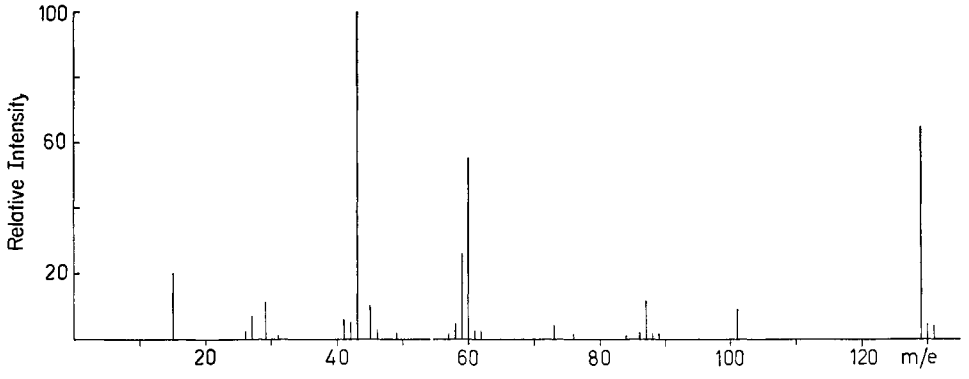
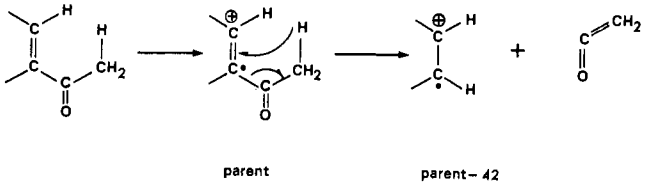


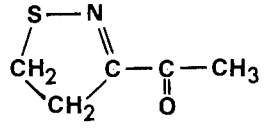
Figure 2. Mass spectrum of 2-acetyl-2-thiazoline

mass spectrum, an unsaturated cyclic structure seems likely. The combination of infrared absorptions at 1700, 1357, and 1262 cm^{-1} with those at 608 and 549 cm^{-1} (Katon and Bentley, 1963) is strong evidence for the presence of an acetyl group. The rather low value of the carbonyl stretching frequency (1700 cm^{-1}) indicates that the acetyl group is either conjugated with a double bond or is subject to severe steric hindrance. The occurrence of a peak at m/e = 87 in the mass spectrum is also significant; a fragment with mass parent-42 is a common feature in the spectra of compounds which have an acetyl group attached to a double bond. Most likely these ions originate through the following rearrangement reaction.

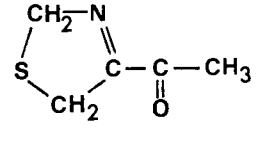


The infrared spectrum showed the absence of any absorption bands attributable to the functional groups NH and SH. Since the oxygen atom is accounted for in the acetyl group, the nitrogen and sulfur atoms must be situated in the ring.

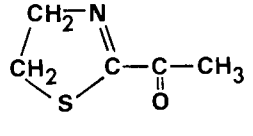
The three structures which fit our spectral data are shown below.



3-acetyl-2-isothiazoline

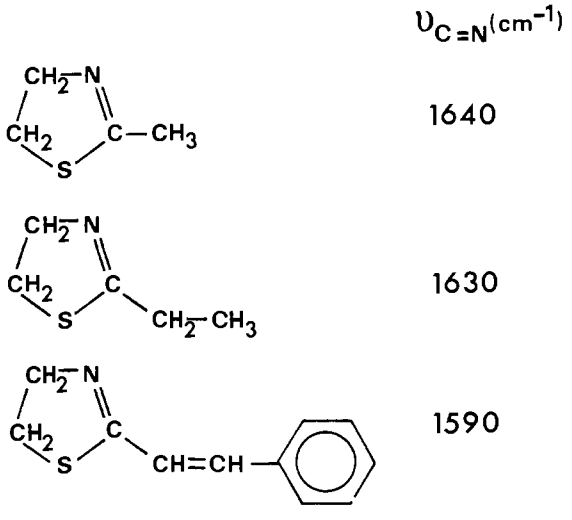


4-acetyl-3-thiazoline



2-acetyl-2-thiazoline

Asinger and Gluch (1961) showed that the C=N stretching frequency of 3-thiazolines usually occurs near 1670 cm^{-1}. Although conjugation with the acetyl group would lower this frequency by some 20 to 40 cm^{-1}, it seems very unlikely that the observed value of 1589 cm^{-1} can be accounted for in this way. Otting and Drawert (1955) found that 2-thiazolines have a strong absorption band near 1640 cm^{-1}. This low value is ascribed to conjugation of lone-pair sulfur electrons with the π-electrons of the double bond. Further conjugation with a second double bond brings the C=N stretching frequency in these compounds well below 1600 cm^{-1}.



The combined spectral evidence points to 2-acetyl-2-thiazoline. That we were unable to detect a significant absorption in the uv at the given concentration (a few μg per ml) and the applied cell length of 1 cm is explained by the fact that 2-acetyl-2-thiazoline has a molar extinction coefficient of only 3000 l./mol cm (Asinger and Gluch, 1961). Final proof of the identity was obtained by comparison of the properties of the unknown with those of a synthesized sample of 2-acetyl-2-thiazoline.

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