COMMUNICATIONS

On the Amounts of Urethane Formed in Diethyl Pyrocarbonate Treated Beverages

Experimental evidence is presented to show that by reaction kinetic calculations the highest limit of the amount of urethane formed in ammonia-containing beverages treated with diethyl pyrocarbonate under defined conditions can be predicted. This offers a simple and unequivocal way of deciding whether or not a wine sample of a given pH and ammonia content can be treated with diethyl pyrocarbonate so as not to exceed the permissible level of urethane, 10 μg/liter.

Diethyl pyrocarbonate, $(EtOCO)_2O$, is known to be a potent sterilizing agent. As an electrophile, it reacts with nucleophilic groups of essential biomolecules, mainly proteins (Ehrenberg et al., 1976). Such a reaction results in the inactivation of enzymes and other biologically important cell constituents and, ultimately, leads to a killing of germs (Pauli and Genth, 1966). In a concomitant reaction with water, $(EtOCO)_2O$ is rapidly decomposed:

$$(EtOCO)_2O + H_2O \xrightarrow{k'} 2CO_2 + 2EtOH \quad (1)$$

Since the products of decomposition, ethanol and carbon dioxide, are nontoxic, natural compounds, $(EtOCO)_2O$ seems to be an ideal preservative.

Indeed, $(EtOCO)_2O$ has been used successfully as a cold sterilizing agent of wine and juice until warnings by Gejvall and Löfroth (1971) and Löfroth and Gejvall (1971) focused attention to an earlier observation of Pauli and Genth (1966) that reaction of $(EtOCO)_2O$ with ammonia leads to the formation of the carcinogenic urethane:

$$(EtOCO)_2O + NH_3 \xrightarrow{k_2} EtOCONH_2 + CO_2 + EtOH \quad (2)$$

Since beverages, like any biological material, contain ammonia, their treatment with $(EtOCO)_2O$ will lead to urethane formation. The amount of urethane formed this way is critical for the admittance of $(EtOCO)_2O$ as a cold sterilizing agent of beverages. Therefore, quantitative determinations of urethane in $(EtOCO)_2O$-treated materials were carried out in a number of laboratories. Unfortunately, earlier literature data concerning the amounts of urethane formed in $(EtOCO)_2O$-treated beverages and buffers varied to a considerable extent.

Recently, Ough (1976b) reinvestigated the formation of urethane in $(EtOCO)_2O$-treated wine. Using an accurate gas chromatographic method for the quantitative determination of urethane he found, in accordance with Fischer (1972), that the urethane concentration is about two orders of magnitude lower than that reported by Gejvall and Löfroth (1971) and Löfroth and Gejvall (1971) for wine samples treated with $(EtOCO)_2O$ under the same conditions. Although Ough's data are convincing enough to be accepted, the experiments carried out by Ough (1976b), just as by others (Gejvall and Löfroth, 1971; Löfroth and Gejvall, 1971; Fischer, 1972), were complicated by difficulties involved in the determination of minute amounts of urethane.

In the present paper it will be shown that by reaction kinetic calculations the amounts of urethane formed in $(EtOCO)_2O$-treated beverages can be predicted. The validity of these calculations will be demonstrated in experiments based on a reliable test involving the determination of ammonia in the sample both before and after $(EtOCO)_2O$ treatment. In this way the cumbersome method of urethane determination is also avoided.

MATERIALS AND METHODS

$(EtOCO)_2O$ under the trade name "Baycovin" was obtained from Bayer, Leverkusen. All inorganic chemicals, of analytical grade, were purchased from Merck, Darmstadt, and organic compounds from Sigma, St. Louis, Mo. The wine was a bottled brand "Val de Loire" commercially available in Sweden.

For the estimation of the ammonia content of buffers and wine, the chemicals provided in a kit by Hyland Laboratories, Costa Mesa, Calif., were used: Ammonia was selectively adsorbed to an ion-exchange resin, followed by elution with 4 M NaCl and determined as ammonia nitrogen by the phenate hypochlorite method as described in a brochure of Hyland Laboratories (1973).

The pseudo-first-order rate constant, k', for the hydrolysis of $(EtOCO)_2O$ in 0.05 M acetate, pH 4.75, as well as in the above buffer containing 10% w/v ethanol, was determined by estimating the decrease of concentration of $(EtOCO)_2O$ at different times and temperatures by the imidazole method (Osterman-Golkar et al., 1974) and the half-life values, $t_{1/2}$, of $(EtOCO)_2O$ obtained this way were substituted into the expression $k' = 0.693/t_{1/2}$. Since k' for the hydrolysis of $(EtOCO)_2O$ has been shown not to depend on pH in the acidic range (Larrouquère, 1965), only the effects on k' of temperature and ethanol content of the medium were measured.

The second-order rate constants, k_2, for the reaction of $(EtOCO)_2O$ with ammonia were determined by estimating the amounts of ammonia consumed by $(EtOCO)_2O$ at different temperatures, after the hydrolysis of $(EtOCO)_2O$ went to completion in 10 mL of 0.05 M acetate, pH 4.75, containing 3.96 mmol of ammonia and 3.32 mmol of $(EtOCO)_2O$ and by substituting the values obtained into eq 4.

Treatment of wine or buffer with $(EtOCO)_2O$ was carried out by adding given amounts of $(EtOCO)_2O$ to the sample and shaking the mixture for 48 h at the temperature indicated.

RESULTS AND DISCUSSION

In the experiments shown in Table I, wine and buffers containing a given amount of ammonia were treated at different pH values and temperatures with various amounts of $(EtOCO)_2O$, and the ammonia content of the samples was determined both before and after $(EtOCO)_2O$ treatment. The difference in ammonia content was taken to be equivalent with the amount of urethane formed in the sample:

0021-8561/78/1426-0500$01.00/0

Table I. Comparison of Theoretically Expected and Experimentally Found Concentrations of Urethane Formed in Wine and Buffer upon Treatment with Diethyl Pyrocarbonate

No.	Reaction medium	Temperature, °C	pH	$[NH_3{}^+NH_4{}^+]_0$, M	$[(EtOCO)_2O]_0$,[a] M	k_2, L mol^{-1} min^{-1}	k', min^{-1}	[Urethane] expected, M	[Urethane] found, M
1	Wine, Val de Loire	23	3.80	4.71×10^{-4}	2.10×10^{-1}	2.54×10^{3}	2.29×10^{-2}	4.4×10^{-5}	1.6×10^{-5}
2	Wine, Val de Loire	23	3.80	4.71×10^{-4}	4.15×10^{-1}	2.54×10^{3}	2.29×10^{-2}	8.2×10^{-5}	5.7×10^{-5}
3	Wine, Val de Loire	23	3.80	4.71×10^{-4}	6.25×10^{-1}	2.54×10^{3}	2.29×10^{-2}	11.8×10^{-5}	9.3×10^{-5}
4	Wine, Val de Loire	23	3.80	4.71×10^{-4}	8.30×10^{-1}	2.54×10^{3}	2.29×10^{-2}	15.0×10^{-5}	8.1×10^{-5}
5	Wine, Val de Loire	23	3.80	4.71×10^{-4}	1.25	2.54×10^{3}	2.29×10^{-2}	2.1×10^{-4}	1.5×10^{-4}
6	Wine, Val de Loire, pH adjusted with NaOH	23	4.73	4.50×10^{-4}	3.32×10^{-1}	2.54×10^{3}	2.29×10^{-2}	3.3×10^{-4}	2.2×10^{-4}
7	Wine, Val de Loire, pH adjusted with NaOH	5	4.73	4.50×10^{-4}	3.32×10^{-1}	1.09×10^{3}	5.02×10^{-3}	2.2×10^{-4}	1.5×10^{-4}
8	Acetate, 0.05 M, with 10% w/v ethanol	23	4.93	3.96×10^{-4}	3.32×10^{-1}	2.54×10^{3}	2.29×10^{-2}	3.5×10^{-4}	3.4×10^{-4}
9	Acetate, 0.05 M, with 10% w/v ethanol	5	4.93	3.96×10^{-4}	3.32×10^{-1}	1.09×10^{3}	5.02×10^{-3}	2.6×10^{-4}	2.4×10^{-4}

[a] Concentrations of $(EtOCO)_2O$ which are higher than the saturating concentration, 40 mM, refer to a total concentration obtained by repeated additions of saturating amounts of $(EtOCO)_2O$ to the system.

$$[EtOCONH_2] = [NH_3 + NH_4{}^+]_0 - [NH_3 + NH_4{}^+]_{final} \quad (3)$$

The respective values are represented in Table I under the heading Urethane Found. Table I also shows the theoretically expected urethane concentrations in the samples. These values were calculated from eq 4 which describes the expected change in ammonia concentration after $(EtOCO)_2O$ treatment in terms of an exponential function of e:

$$[NH_3 + NH_4{}^+]_{final}/[NH_3 + NH_4{}^+]_0 = e^{-k_2 b[(EtOCO)_2O]k'} \quad (4)$$

where square brackets indicate the concentrations of the compounds involved, k' is the pseudo-first-order rate constant of the hydrolysis of $(EtOCO)_2O$, k_2 the second-order rate constant of the reaction shown in eq 2, and b is the fraction of ammonia present in nonprotonated, reactive form, i.e., b = antilog (pH − pK). The pK_a values of ammonia, 9.9 and 9.3 at 5 and 23 °C, respectively (Sober, 1970), were reduced by 1.3% (Bates, 1973) to be applicable to wine or aqueous solutions containing 10% w/v ethanol.

As seen from the data in Table I, the amounts of urethane found in $(EtOCO)_2O$-treated buffer are closer to the calculated values than those obtained with wine. This suggests that owing to some interfering reaction(s) urethane formation is somewhat suppressed in wine. Thus, our calculations overestimate rather than underestimate the amounts of urethane formed in $(EtOCO)_2O$-treated wine containing ammonia.

In Table II the expected concentrations of urethane are compared with the concentrations found by Löfroth and Gejvall (1971), Fischer (1972), and Ough (1976b). Since in the experiments of the above authors the conditions were such (acidic pH, low concentration of $(EtOCO)_2O$) as to permit the formation of only low amounts of urethane, the concentration of ammonia can be considered constant throughout the reaction. In such cases eq 3 and eq 4 can be replaced by eq 5 to give a satisfactory approximation of the amount of urethane to be expected:

$$[EtOCONH_2] = k_2[NH_3 + NH_4{}^+]b\frac{[(EtOCO)_2O]}{k'} \quad (5)$$

It can be seen that an acceptable agreement exists between the expected and found amounts of urethane, except some data obtained by Löfroth and Gejvall (1971). These authors overestimated the quantity of urethane formed during $(EtOCO)_2O$ treatment in orange juice, beer, and wine by a factor of 10, 70, and 300, respectively (sample No. 1, 6, and 7, Table II).

By applying our way of calculation one can predict that in an average wine (pH 3.44, 0.205 mM ammonia, see sample 24 in Table II) upon treatment with 1.85 mM $(EtOCO)_2O$ (300 ppm as suggested by the World Health Organization (1967) for the cold sterilization of beverages) no more urethane will be generated than 0.068 μM, i.e., 6.05 μg of urethane/liter. This concentration does not exceed that (10 μg/liter) considered by the World Health Organization, Joint FAO/WHO Expert Committee on Food Additives (1972) to be permissible.

Nevertheless, due to a disagreement of views concerning the amounts of urethane formed in $(EtOCO)_2O$-treated beverages on the one hand, and a lack of an easily applicable method of urethane determination on the other, the use of $(EtOCO)_2O$ as a cold sterilizer of beverages has not been permitted by certain authorities. A recent study of Ough (1976a) cast some doubt on the adequacy of such a decision: he has shown that urethane is a naturally occurring component of fermented foods and beverages, and amounts to about 1.5 μg in 1 kg of bread and from 1.3

Table II. Urethane Concentrations Found by Different Authors in Various Solutions and Beverages Treated with Diethyl Pyrocarbonate as Compared with Theoretically Expected Concentrations of Urethane

No.	Reaction medium	Temperature, °C	pH	$[NH_3^+NH_4^+]_0$, M	$[(EtOCO)_2O]_0$, M	k_2, L mol^{-1} min^{-1}	k', min^{-1}	[Urethane] expected, M	[Urethane] found, M	Reference
1	Orange juice	6	3.80	1.18×10^{-3}	1.73×10^{-3}	2.66×10^{2}	7.71×10^{-3}	2.6×10^{-7}	20.2×10^{-7}	*c*
2	Orange juice, pH adjusted with NaOH[a]	6	4.50	1.18×10^{-3}	1.73×10^{-3}	2.66×10^{2}	7.71×10^{-3}	1.3×10^{-6}	2.3×10^{-6}	*c*
3	Orange juice, pH adjusted with NaOH	6	4.50	1.18×10^{-3}	3.46×10^{-3}	2.66×10^{2}	7.71×10^{-3}	2.6×10^{-6}	5.2×10^{-6}	*c*
4	Orange juice, pH adjusted with NaOH	6	4.50	1.18×10^{-3}	6.92×10^{-3}	2.66×10^{2}	7.71×10^{-3}	5.1×10^{-6}	6.5×10^{-6}	*c*
5	Orange juice, pH adjusted with NaOH	6	4.50	4.12×10^{-3}	1.73×10^{-3}	2.66×10^{2}	7.71×10^{-3}	4.5×10^{-6}	4.0×10^{-6}	*c*
6	Beer	6	4.40	1.18×10^{-4}	3.46×10^{-3}	2.66×10^{2}	7.71×10^{-3}	2.1×10^{-7}	1.5×10^{-5}	*c*
7	Wine, white	6	3.40	2.94×10^{-4}	3.46×10^{-3}	2.66×10^{2}	5.48×10^{-3}	9.4×10^{-8}	2.9×10^{-5}	*c*
8	Aqueous solution	15	3.00	1.67×10^{-3}	1.85×10^{-3}	8.32×10^{2}	1.67×10^{-2}	8.9×10^{-8}	11.0×10^{-8}	*d*
9	Aqueous solution	15	3.50	1.67×10^{-3}	1.85×10^{-3}	8.32×10^{2}	1.67×10^{-2}	2.8×10^{-7}	3.4×10^{-7}	*d*
10	Aqueous solution	15	4.00	1.67×10^{-3}	1.85×10^{-3}	8.32×10^{2}	1.67×10^{-2}	8.9×10^{-7}	9.7×10^{-7}	*d*
11	Orange juice	15	3.50	1.29×10^{-3}	1.85×10^{-3}	8.32×10^{2}	1.67×10^{-2}	2.2×10^{-7}	1.6×10^{-7}	*d*
12	Grapefruit juice	15	3.00	9.41×10^{-4}	1.85×10^{-3}	8.32×10^{2}	1.67×10^{-2}	5.0×10^{-8}	13.5×10^{-8}	*d*
13	Wine, Mosel	15	3.20	1.47×10^{-3}	1.85×10^{-3}	8.32×10^{2}	1.18×10^{-2}	2.3×10^{-7}	4.5×10^{-7}	*d*
14	Buffer[b] with 11% v/v ethanol	20	3.00	2.94×10^{-3}	1.23×10^{-3}	1.52×10^{3}	1.78×10^{-2}	2.3×10^{-7}	2.0×10^{-7}	*e*
15	Buffer[b] with 11% v/v ethanol	20	3.00	2.94×10^{-3}	2.47×10^{-3}	1.52×10^{3}	1.78×10^{-2}	4.7×10^{-7}	4.9×10^{-7}	*e*
16	Buffer[b] with 11% v/v ethanol	20	3.50	2.94×10^{-3}	1.23×10^{-3}	1.52×10^{3}	1.78×10^{-2}	7.4×10^{-7}	4.4×10^{-7}	*e*
17	Buffer[b] with 11% v/v ethanol	20	3.50	2.94×10^{-3}	2.47×10^{-3}	1.52×10^{3}	1.78×10^{-2}	14.8×10^{-7}	9.2×10^{-7}	*e*
18	Buffer[b] with 11% v/v ethanol	20	4.00	2.94×10^{-3}	1.23×10^{-3}	1.52×10^{3}	1.78×10^{-2}	2.3×10^{-6}	2.0×10^{-6}	*e*
19	Buffer[b] with 11% v/v ethanol	20	4.00	2.94×10^{-3}	2.47×10^{-3}	1.52×10^{3}	1.78×10^{-2}	4.7×10^{-6}	3.9×10^{-6}	*e*
20	Wine, Chardonnay, ammonia added	20	3.08	6.76×10^{-3}	1.23×10^{-3}	1.52×10^{3}	1.78×10^{-2}	6.5×10^{-7}	5.7×10^{-7}	*e*
21	Wine, Ruby Cabernet, ammonia added	20	3.40	6.76×10^{-3}	1.23×10^{-3}	1.52×10^{3}	1.78×10^{-2}	13.6×10^{-7}	8.0×10^{-7}	*e*
22	Wine, Malbec, ammonia added	20	3.88	6.82×10^{-3}	1.23×10^{-3}	1.52×10^{3}	1.78×10^{-2}	4.1×10^{-6}	1.2×10^{-6}	*e*
23	Wine, Tinto Cao, ammonia added	20	4.40	1.00×10^{-2}	1.23×10^{-3}	1.52×10^{3}	1.78×10^{-2}	20.1×10^{-6}	5.3×10^{-6}	*e*
24	Average wine[f]	20	3.44	2.05×10^{-4}	1.85×10^{-3}	1.52×10^{3}	1.78×10^{-2}	6.8×10^{-8}	3.4×10^{-8}	*e*

[a] Average values of three different samples treated under the same conditions. [b] 0.2 M K_2HPO_4 + 0.1 M citric acid to give the pH indicated. [c] Löfroth and Gejvall, 1971. [d] Fischer, 1972. [e] Ough, 1976b. [f] Average values from Table II of Ough's paper (Ough, 1976b).

to 4.9 μg in 1 L of wine. In view of the above data and arguments, we feel that instead of simply banishing $(EtOCO)_2O$ from beverage industry, prescribing the conditions of its use on a scientific basis would be more reasonable. We suggest that before treatment the ammonia content and the pH of the individual samples be determined, eq 5 be consulted, using the proper values for k' and k_2 (see Tables I and II), and a decision as to the controlled use of $(EtOCO)_2O$ be made depending on the expected amount of urethane generated under the given conditions.

ACKNOWLEDGMENT

We thank L. Ehrenberg and Siv Osterman-Golkar, University of Stockholm, as well as L. Maros and F. Ruff, Eötvös University, Budapest, for helpful discussions.

LITERATURE CITED

Bates, R. G., "Determination of pH, Theory and Practice", Wiley, New York, N.Y., 1973, p 222.
Ehrenberg, L., Fedorcsák, I., Solymosy, F., *Prog. Nucleic Acid Res. Mol. Biol.* **16**, 189 (1976).
Fischer, E., *Z. Lebensm.-Untersuch. Forsch.* **148**, 221 (1972).
Gejvall, T., Löfroth, G., *Newsl. Environ. Mutagen Soc.* **5**, 35 (1971).
Hyland Laboratories, "Blood Ammonia Test", 1973.
Larrouquère, J., *Bull. Soc. Chim. Fr.*, 382 (1965).
Löfroth, G., Gejvall, T., *Science* **174**, 1248 (1971).
Osterman-Golkar, S., Ehrenberg, L., Solymosy, F., *Acta Chem. Scand. Ser. B* **28**, 215 (1974).
Ough, C. S., *J. Agric. Food Chem.* **24**, 323 (1976a).
Ough, C. S., *J. Agric. Food Chem.* **24**, 328 (1976b).
Pauli, O., Genth, H., *Z. Lebensm.-Untersuch. Forsch.* **132**, 216 (1966).
Sober, H. A., Ed., "Handbook of Biochemistry", The Chemical Rubber Co., Cleveland, Ohio, 1970, p J-74.
World Health Organization, "Food Additives", No. 67.29, 1967, pp 34–38.
World Health Organization, "Joint FAO/WHO Committee on Food Additives", Series No. 4, 1972, pp 67–74.

F. Solymosy*
F. Antoni
I. Fedorcsák

Institute of Biochemistry 1
Semmelweis University Medical School
Puskin u.9
1088 Budapest, Hungary

Received for review March 24, 1977. Accepted October 4, 1977. This work has been supported by the Royal Swedish Academy of Sciences, the Swedish Atomic Research Council and the Hungarian Academy of Sciences.

Effects of Blanching on Protein Fractions of Certain Sweet Corn Cultivars

Blanching of sweet corn did not alter significantly total protein, alkali-soluble protein, or free amino acids. Blanching reduced salt and alcohol-soluble proteins and caused marked increases of insoluble protein. Changes during blanching varied with cultivars and stage of maturity. Attention to those variables when selecting sweet corn for processing could help improve product quality.

When heated under moist conditions, many proteins are denatured and become much less soluble in aqueous media. Seed proteins differ considerably from animal proteins in that plant proteins often are incompletely coagulated by heat (Osborne, 1924). Heat sensitivity of corn globulins is influenced by moisture content, temperature, and duration of heating (Rukina and Ruchkin, 1970). Rukina and Ruchkin (1970) reported that heat decreased the salt-soluble protein fraction of field corn. Green (1972) found that heated sweet corn had a lower sulfhydryl content in the salt-soluble fraction, and he concluded that soluble proteins influenced the physical characteristics of processed sweet corn.

Quantities of certain protein fractions of sweet corn vary among cultivars and with stage of maturity (Pukrushpan et al., 1977). The objective of this study was to determine the effects of blanching on the protein fractions of the cultivars when harvested at various stages of maturity.

MATERIAL AND METHODS

Five sweet corn cultivars were grown, harvested, and sampled as described by Pukrushpan et al. (1977). Samples with more than 80% moisture were classified as immature, those between 75 and 77% as mature, and those with 70 to 72% moisture as overmature. The ears were blanched in boiling water for 6 min and were cooled by immersing in cold water. The ears were drained, packaged in polyethylene bags, and stored at −20 °C. The frozen corn was prepared for analysis by dipping in boiling water for 3 min, cutting the kernels from the cob, and homogenizing the kernels in acetone to yield a defatted residue. Protein fractionation and analyses were performed as described by Pukrushpan et al. (1977).

The data were analyzed statistically by the analysis of variance, F test, and least significant differences (LSDs).

RESULTS AND DISCUSSION

In earlier work by Pukrushpan et al. (1977), certain protein fractions varied significantly with stage of maturity and among cultivars. In this experiment, protein fractions of unblanched corn again differed significantly with stage of maturity, but only total protein and free amino acids differed significantly among cultivars (Table I). However, stages of maturity at time of sampling were different from those of the earlier report.

Total protein concentration decreased with increasing maturity (immature 16.5, mature 14.0, overmature 12.6%, LSD 0.9) and varied with cultivars (Bonanza 15.68 Jubilee 15.2, Triumphant II 14.2, Yukon 13.2, and NK51036 13.0%, LSD 1.2). Salt-soluble protein decreased with increasing maturity (immature 4.5, mature 3.1, overmature 2.4%, LSD 0.7). Alcohol-soluble protein varied significantly only with stage of maturity (immature 1.9, mature 3.1, overmature 3.8%, LSD 0.5). Alkali-soluble protein also varied only with maturity (immature 4.8, mature 4.2, overmature 3.9%, LSD 0.7), as did insoluble protein (immature 3.6, mature 2.3, overmature 1.8%, LSD 0.6). Free amino acids differed both by cultivar and with maturity (Bonanza 0.22, Jubilee 0.15, NK51036 0.15, Triumphant II 0.13, Yukon 0.13%, LSD 0.04; immature

0021-8561/78/1426-0503$01.00/0