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## Ethylcarbamate in Fermented Beverages and Foods.

### II. Possible Formation of Ethylcarbamate from Diethyl Dicarboxylate Addition to Wine

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Determination was made of the effects on the formation of ethylcarbamate in model solutions that pH, ammonia, and diethyl dicarboxylate (DEDC) have in the ranges found or used in acidic beverages. Prediction of the maximum possible amount of ethylcarbamate that can be formed is expressed graphically in terms of the three variables. DEDC added to wines forms less than the amounts predicted because of other competitive reactions. The amounts of ethylcarbamate formed in commercial wines, under controlled conditions, are less than 1  $\mu\text{g}/\text{l}$ . The amounts formed under optimum industrial conditions are below the sensitivity of the test to detect. These data indicate that the compound's FDA status should be reevaluated.

A preceding article (Ough, 1976) discussed the recent history of diethyl dicarboxylate (DEDC) and the reasons for its discontinuance as an additive to beverages and foods. The results reported in that paper clearly indicate that ethylcarbamate is a natural component in wine and is probably present in all fermented foods and beverages.

This article describes tests made on model solutions and on wines in which the parameters that affect the formation of ethylcarbamate when DEDC is added were controlled or known.

#### EXPERIMENTAL SECTION

Extraction, concentration, and analysis by gas chromatography with the Coulson detector have been described (Ough, 1976).

$\text{NH}_3$  analyses were performed by specific ion electrode techniques as described by McWilliam and Ough (1974).

Since the formation of ethylcarbamate from added DEDC would have to occur from its reaction with ammonia, an experiment was designed to determine the degree to which it could be formed in model systems without competing reactions. These levels would then represent the maximum obtainable with the pH,  $\text{NH}_3$ , and DEDC levels investigated.

**pH-Ammonia-DEDC Parameters for Ethylcarbamate Formation.** Buffer solutions were made with 0.2 M  $\text{K}_2\text{HPO}_4$  and 0.1 M citric acid, both in 11% v/v ethanol. Precalculated amounts were mixed to give a series of solutions with pH values of 3.0, 3.25, 3.50, 3.75, and 4.00. Final adjustments were made with concentrated NaOH or  $\text{H}_2\text{SO}_4$ . Each pH solution was subdivided into four sublots, and the ammonia content of each was adjusted to 0, 50, 100, and 200 mg/l. Each of these samples was subdivided further into five lots, and DEDC (obtained from Bayer AG and checked for purity, exceeding 99.5%) was added at 0, 50, 100, 200, and 400 mg/l. Samples were stored for 72 h at 20 °C. The samples were analyzed by saturating 250

ml with 70 g of NaCl and extracted three times each with 100 ml of ethyl acetate; the extracts were combined, dried over  $\text{Na}_2\text{SO}_4$ , and reduced in volume as described previously (Ough, 1976). A standard curve was prepared. Peak responses of samples were compared with that of a similar external standard using a 3% Versamide liquid phase on Chromosorb G 60-80 acid washed (column A as described by Ough, 1976). Recovery from model solutions was consistent and in excess of 80%. Measurements of peak heights gave linear response over a wide range. The amounts of ethylcarbamate produced at various combinations were calculated from a standard curve and external standards.

The data (Table I) show the amounts of ethylcarbamate produced in the various combinations of variables. The data are further displayed in the plot of Figure 1 (log of the micrograms of ethylcarbamate produced/milliliter of ammonia present compared with the pH), indicating a linear response.

Since these data permitted prediction of the maximum amounts of ethylcarbamate that could be formed under the conditions described, a number of wine samples representative of a broad spectrum of commercial products were treated with various levels of DEDC and analyzed for any ethylcarbamate that might result.

Two hundred milligrams of DEDC (Bayer AG-purity 99.5%) was added per liter to a number of wines (prepared at the University of California Enology Laboratory). After 72 h or more at 20 °C  $\text{NH}_3$  at about 100 mg/l. was added to samples of some of the same wines, and DEDC was also added. Wines were analyzed for original pH and  $\text{NH}_3$  content.

These data are shown in Table II. In addition, analysis of the ethylcarbamate in these wines is reported and plotted in Figure 2. Measurement was as reported previously (Ough, 1976). The lower limit of detection is about 2.5 ng/injection (threefold to fivefold more peak height than baseline variation). The reproducibility of the method, measured on 19 replicated samples ranging from 1.6 to 5.2  $\mu\text{g}/\text{l}$ ., reported as relative standard deviation, is

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Table I. Ethylcarbamate Produced ( $\mu\text{g/l.}$ ) at Several pH Values, Concentrations of Ammonia, and DEDC

Diethyl pyro-carbonate, mg/l.	pH														
	3.0			3.25			3.50			3.75			4.00		
	NH <sub>3</sub> , mg/l.			NH <sub>3</sub> , mg/l.			NH <sub>3</sub> , mg/l.			NH <sub>3</sub> , mg/l.			NH <sub>3</sub> , mg/l.		
	50	100	200	50	100	200	50	100	200	50	100	200	50	100	200
50	4	10	10	4	16	19	12	21	34	36	54	106	30	69	140
100	9	11	17	8	15	27	19	32	70	58	103	179	70	152	320
200	18	30	40	24	35	87	39	48	187	90	124	323	180	330	705
400	44	71	135	69	133	300	82	144	322	216	364	927	350	750	1400

Table II. Effect of Added Ammonia on Ethylcarbamate Formation in Wines by DEDC

Variety	Region	pH	Total NH <sub>3</sub> , mg/l.		Ethylcarbamate formed, <sup>a</sup> $\mu\text{g/l.}$	
			Control	Added	Control	Added
Pinot noir	Oakville	3.13	3.2	115	0.6	30, 19
Ruby Cabernet	Kearney	3.40	2.8	115	1.7	71
French Colombard	Davis	3.10	11.0	123	5.3	37
White Riesling	Oakville	2.88	2.4	114	0.4	32
Chardonnay	Oakville	3.08	3.3	115	2.7	56, 45
French Colombard	Kearney	3.14	2.9	115	0.4	33, 51
Cabernet Sauvignon	Oakville	3.12	1.3	113	1.6	36
Thompson Seedless	Kearney	2.93	3.4	115	0.9	21
Barbera	Kearney	3.35	2.3	114	2.3	65
Malbec	Hopland	3.88	3.5	116	4.5	102
Petite Sirah	Rancho, Calif.	3.86	2.2	114	1.9	42
Cabernet Sauvignon	Rancho, Calif.	3.86		114		46 <sup>b</sup>
Pinot blanc	Rancho, Calif.	3.58		114		88 <sup>b</sup>
Grey Riesling	Rancho, Calif.	3.68		115		104 <sup>b</sup>
Chenin blanc	Rancho, Calif.	3.58		112		101 <sup>b</sup>
Tinto Cao	Kearney	4.40		170		470 <sup>b</sup>

<sup>a</sup> Upon addition of DEDC at 200 mg/l., "added" column corrected for amount of ethylcarbamate in the untreated control. <sup>b</sup> Not corrected for original ethylcarbamate in the control.

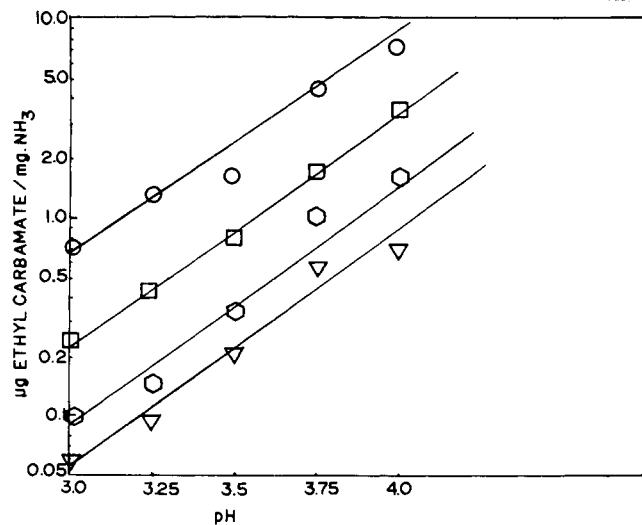


Figure 1. Relationship of amount of ethylcarbamate (per unit of ammonia) formed by the addition of various DEDC concentrations (mg/l.) (50 ( $\nabla$ ); 100 ( $\circ$ ); 200 ( $\square$ ); 400 ( $\circ$ )) at various pH values in model solutions.

about 21%. As long as the solvents, chloroform and ethyl acetate, were properly redistilled, no interfering solvent peaks were noted. Solvent blanks, continued throughout the procedure, were checked at intervals.

Table III shows the results of ethylcarbamate analysis of 15 commercial wine samples treated with practical amounts of DEDC and samples of the same wines fortified with ethylcarbamate at 10  $\mu\text{g/l.}$

#### DISCUSSION

##### Formation of Ethylcarbamate from Added DEDC.

Table II summarizes the amounts of ethylcarbamate found in the DEDC-treated control wines compared with the wines treated first with ammonia and then with DEDC.

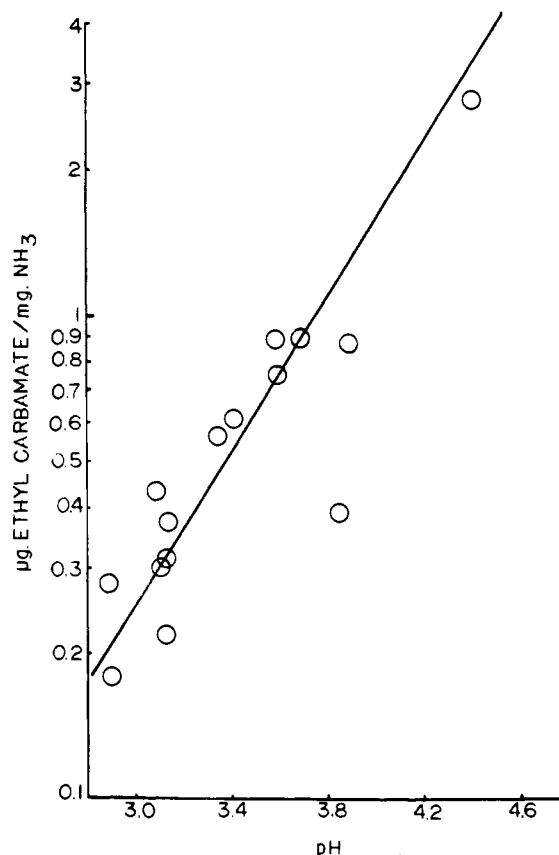


Figure 2. The formation of ethylcarbamate (per unit of NH<sub>3</sub> present) from the addition of DEDC (200 mg/l.) to wine as related to the pH of the wine.

The increases in ethylcarbamate levels were small but measurable in the wines containing low levels of ammonia but were significant when ammonia was high. The effect

Table III. Ethylcarbamate Determined in Commercial Wines Treated with Diethyl Dicarbonate (DEDC) and Recovery of Added Ethylcarbamate

Wine types	pH	NH <sub>3</sub> , mg/l.	Ethylcarbamate, $\mu\text{g/l.}$					
			DEDC added, mg/l.				With ethyl- carbamate added	% recovery
			Control	50	75	100		
White wines								
Special natural	3.4	0.2	1.4	0.0	1.2	1.0	9.0	76
Special natural	3.2	0.3	2.5	2.8	5.0	3.9	7.6	51
Rhine	3.0	3.3	0.8	1.2	1.5	1.3	9.6	88
Special natural	3.3	4.5	1.4	1.4	0.9	1.5	5.9	45
Special natural	3.2	1.4	1.2	6.2	1.4	4.3	5.6	44
Special natural	3.1	15.0	1.9	1.4, 4.1, 0.0			10.0	81
Riesling	3.1	9.8	3.1	3.7, 4.6	3.4		11.4	83
Pink Wines								
Rosé	3.0	5.7	1.4	1.3	1.0	1.8	10.8	94
Rosé	3.2	3.2	0.3	0.3	0.2	0.6	5.9	56
Special natural	3.0	6.0	3.7	4.6	3.8	4.3	12.9	92
Red Wines								
Special natural	3.2	0.3	5.4	6.4	7.0	7.1	15.8	104
Zinfandel	3.3	4.0	1.8	0.7	1.5	0.5	7.0	52
Burgundy	3.3	4.8	1.7	1.8, 1.8, 1.2			13.9	122
Burgundy	3.3	8.0	3.6	3.5, 3.5, 4.1			9.3	57
Chianti	3.6	7.7	1.5	2.0, 1.4, 1.2			5.8	43
Average				0.23	0.35	0.64		72.5

of pH is also indicated. The ratios of ethylcarbamate to the ammonia concentration are plotted in Figures 1 and 2. Clearly the absolute maximum concentration of ethylcarbamate can be reasonably predicted from Figure 1. For example, if a wine has a pH of 3.5 and an ammonia content of 10 mg/l., and DEDC at 100 mg/l., the amount of ethylcarbamate produced can be predicted. The intersection of the 100 mg/l. DEDC line at pH 3.5 and reading of the ethylcarbamate/mg of NH<sub>3</sub> at that intersect gives about 0.35  $\mu\text{g/mg}$  of NH<sub>3</sub>. If the wine has 10 mg/l. of NH<sub>3</sub> then  $10 \times 0.35 = 3.5 \mu\text{g/l.}$  of ethylcarbamate might be produced. The amount produced in wine (Figure 2) indicates a safety factor of some extent at the higher pH values. This may be partially explained by the higher amount of other nitrogen compounds and other basic materials usually present in wine of high pH. Furthermore, the average data determined from the commercial wines show that the amounts formed may be vanishingly small. The method is really not accurately responsive at amounts of less than 1  $\mu\text{g/l.}$  The recovery study shows only about three-fourths of expected amounts at levels of 10  $\mu\text{g/l.}$

**Ammonia in Wines.** Freshly fermented dry wines contain generally small amounts of ammonia. The range found for 29 California wines made at the University of California was 0.7–58 mg/l. (average 10.5 mg/l.). Of the 29 samples, only 7 were over 10.0 mg/l. (Ough, 1969; McWilliam and Ough, 1974). Commercial bottled wines are generally higher in ammonia. The average value for 78 table wines was 16.6 mg/l. (range 5.5–52.0). Fermenting must that is high in ammonia and ends with excessive ammonia can be depleted of this ammonia by removal of most of the yeast at about 6% residual sugar by centrifugation and allowing a new crop to grow. Fermentation temperature has little effect on final ammonia concentration except perhaps at very high values. Fining a wine with bentonite up to 400 mg/l. caused no significant depletion in ammonia content. Bentonite at 1000 mg/l. reduced the ammonia content of a must by about one-third.

Commercial wines are usually sweetened with mute or grape concentrate. Both of these materials have relatively high amounts of ammonia since neither has supported yeast growth and the subsequent removal of most of the ammonia. Ion-exchange treatment of grape juice to remove

ammonia and other charged nitrogenous materials can be easily done by strong cation (R<sup>+</sup>) exchange (Du Plessis, 1963).

As well as reducing the amino acid content of the wines and thus inhibiting yeast regrowth, this treatment eliminates some of the possible substrates which could form carboethoxy derivatives with the DEDC, thereby increasing effectiveness and decreasing side products.

Alternatively, the wine can be bentonite fined and refrigerated to remove some of the organic and inorganic nitrogen bases (Zamorani et al., 1973). It should be well within the capabilities of wineries to maintain the concentration of ammonia in finished wines at less than 10 mg/l. and, if necessary, less than 5 mg/l. The specific ion electrode system for measurement of NH<sub>3</sub> is extremely accurate and dependable in the range necessary.

**pH of Wines.** Recent published data (Kissler et al., 1973) show a pH range of 3.24–4.15. The pH values of California commercial wines for several years are given by Ough (1964, 1966) and by Berg and Akiyoshi (1971). The pH values in the first two references were "on-the-shelf" wines, while the last publication dealt with wines which had not been ion-exchanged or commercially bottled. The mean pH values were 3.61 for red wine, 3.46 for rosé, and 3.40 for whites. The maximum pH for the "on-the-shelf" wines was 3.80.

Red wines and wines from warmer climates usually have a higher pH than do white wines or wines from cooler areas. The tendency in the warmer areas is to plant varieties which have higher titratable acid and lower pH. The present practice in many wineries is to reduce the pH either by acidification or by hydrogen cation exchange of the wine. The latter treatment generally gives a desirable pH (see Amerine and Ough, 1974). Certain high-acid varieties are picked early to blend with other wines to raise the acid and lower the pH. The pH of wine is a controllable factor for most wineries.

Results of Lofroth and Gejvall (1971) on the amount of ethylcarbamate produced in wine have been shown by Fischer (1971–1972) to be in error. His work shows Lofroth and Gejvall to be high in their results by a factor of 100 or more. The work reported here confirms Fischer's work and indicates that Lofroth and Gejvall are off by a factor of more than 100 as to wines. The amount of ethyl-

carbamate formed by the reaction of ammonia and DEDC in wine is variable with pH and the concentration of the reactants. The analyses of the 15 commercial wines indicate that under conditions of use, with monitored and adjusted pH and ammonia content, not enough ethylcarbamate is formed to be detected accurately even by this sensitive test. The test can detect added ethylcarbamate at 10 µg/l. with reasonable accuracy, allowing the detection of erroneous additions. The amounts formed are far less than that present naturally. It therefore seems plausible that the controlled use of DEDC as a yeast sterilant should be reconsidered.

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## Identification of Volatile Constituents from Grapes

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The volatile constituents of grapes (varieties Riesling, Traminer, Ruländer, Müller-Thurgau, Scheurebe, Optima, and Rieslaner, harvested 1972-1974) were isolated under enzymic inhibition, enriched by liquid-liquid extraction (pentane-methylene chloride, 2:1), and fractionated on silica gel. Analysis by gas chromatography and coupled GLC-mass spectrometry led to the identification of 225 aroma compounds that included 81 hydrocarbons, 48 acids, 31 alcohols, 23 aldehydes, 18 ketones, 11 esters, and 13 constituents of miscellaneous structures. Eighty-one aroma substances were identified for the first time in a grape aroma. The mass spectral identifications were confirmed by matching retention indices.

It is well known that grapes of different varieties show quite characteristic aromas that are also distinctive of the sort-typical wines. But it must be considered that different biosynthetic pathways are interacting at the formation of the aroma of alcoholic beverages. As Drawert and Rapp (1966) and Drawert (1974) described, with the aid of the biotechnological sequence of wine, different factors play a part in the formation of the total aroma. It could be shown that the volatile aroma substances arise from components of the grapes, the activity of fermentation of sugars, and aroma compounds formed during storage. Furthermore, those reactions that are caused by the technology of winemaking are important, such as, for instance, the formation of C<sub>6</sub>-aldehydes and -alcohols during crushing and at the moment of destruction of the cell structure (Drawert et al., 1973).

In our recent studies we identified more than 200 aroma substances in different wine varieties by means of adsorption, gas chromatography, and mass spectrometry (Drawert et al., 1974; Schreier and Drawert, 1974a,b; Schreier et al., 1974a, 1975). These methods are particularly qualified for a tracer analysis because of their selectivity and sensitivity. We found that numerous aroma compounds occurring only in traces (micrograms per liter) are important for the sort-typical aromas of the different

wines. We applied the same methods for the investigation of the grape aroma. The present study gives a survey of the volatile aroma substances identified by GLC-mass spectrometry in grapes.

## EXPERIMENTAL SECTION

**Materials.** Samples of 30 kg of grapes (varieties Riesling, Traminer, Ruländer, Müller-Thurgau, Scheurebe, Optima, and Rieslaner, harvested 1972-1974 in Rheinpfalz and Franken, West Germany) were investigated.

**Aroma Separation.** Two different methods were used in order to inhibit the enzymes (Drawert et al., 1973). (1) The grapes were crushed by adding methanol (total concentration 66%) with a Stephan type crusher. The methanol-juice mixture was separated immediately in a Hafico type hydraulic press. (2) The grapes were stemmed and crushed with a horizontal type crusher-stemmer under carbon dioxide using a short-time heating procedure (5 sec at 100°C; heat exchanger). The juice was evaporated to a volume of about 3 l. using a thin film evaporator (Centritherm, Alfa-Laval).

**Extraction and Fractionation.** The grape volatiles were extracted from the methanolic solution (method 1) and from the aqueous distillate (method 2) with a pentane-methylene chloride (2:1) mixture. The solvent was removed by distillation through a Vigreux column (20 cm, 40°C) (Drawert and Rapp, 1968; Drawert et al., 1969). The aroma concentrate was then subjected to ascending column chromatography using pentane-ether mixtures as the migrating solvents (Schreier and Drawert, 1974b). A particular fraction (pentane elution) could be divided by preparative GLC separation. The column and gas chro-

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