PREAMBLE

Ethyl carbamate (EC, urethane) is a naturally occurring component of all fermented foods and beverages. Because EC has shown a potential for carcinogenity when administered in high doses in animal tests, the wine industry is interested in reducing EC levels in their products. This advisory contains recommendations drawn from scientific research that are designed to help all winegrape growers, winemakers, and other industry members to minimize the levels of EC in wine. These recommendations are advisory only, and not intended to restrict the freedom and diversity of winemaking styles.

FORMATION OF EC:

To better understand the possible actions we can take to minimize levels of EC in wine, it is necessary to review the basics of the major formation pathway and kinetics:

Arginine, usually one of the most abundant yeast available amino acids in grape juice, is taken up by wine yeast as a nutrient and may be metabolized yielding urea if present in excess amounts. If the urea cannot be further metabolized and accumulates above a critical concentration, yeast strains release it from their cells into the wine during or at the end of fermentation. Urea can spontaneously react with the alcohol in wine to form EC. The chemical reaction between urea and ethanol is exponentially accelerated at elevated temperatures. To a lesser extent citrulline, an amino acid which is not incorporated into yeast protein, and is formed during arginine biosynthesis, can serve as an EC precursor. Lactic acid bacteria can also be a source of citrulline under winemaking conditions. However, the key reaction for EC formation in wine is between urea and ethanol.

PREVENTATIVE ACTIONS:

We have developed the following recommendations towards aspects of EC formation in wine:

1. VITICULTURE .......................................................................................................................... 3
   1. Vineyard Fertilization ........................................................................................................ 3
   2. Cover Crops ..................................................................................................................... 4
   3. Cultivars & Rootstocks ................................................................................................... 4
2. JUICE NUTRIENT STATUS/ADDITIONS .................................................................................. 5
3. YEAST STRAINS .................................................................................................................. 6
4. LACTIC ACID BACTERIA ..................................................................................................... 6
5. UREASE APPLICATION ....................................................................................................... 7
6. SUR LIE AGING ................................................................................................................ 7
7. DISTILLATION/FORTIFICATION .......................................................................................... 8
8. SHIPMENT AND STORAGE ............................................................................................... 8

SUMMARY .................................................................................................................................... 9

BIBLIOGRAPHY ......................................................................................................................... 10
VITICULTURE

1 Vineyard Fertilization
• Nitrogen (N) fertilization in the vineyard has direct influence on the nitrogen contents of the grape berry and the resulting must. Excessive fertilization with urea, ammonia and other N-fertilizers in the past is considered partly responsible for generally higher EC levels found in wines from traditional wineproducing countries.

• The following OIV Expert Committee on Vine Physiology method for sampling of leaves to determine the nutritional status of grapevines in order to provide sufficient, but not excessive, nutrients for vine growth has been developed (Resolution Viti 4/95).

There appears to be a growing opinion among viticulturists that nitrate-N in petioles at bloom has little if any relationship to growth, fruitset or general N status of the vine or N status of the berries and resulting juice.

<table>
<thead>
<tr>
<th>OPERATION</th>
<th>LEAF BLADE</th>
<th>PETIOLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Timing</td>
<td>- Berry set</td>
<td>- Véraison</td>
</tr>
<tr>
<td></td>
<td>- Véraison</td>
<td></td>
</tr>
<tr>
<td>Minimum number of vines¹</td>
<td>50-100</td>
<td>100</td>
</tr>
<tr>
<td>Position of the leaf on the shoot</td>
<td>Leaf blade opposite the first basal cluster</td>
<td>Petioles opposite the cluster</td>
</tr>
<tr>
<td>Position of the shoot on the cordon or cane²</td>
<td>Fruitful shoot in the middle</td>
<td>Fruitful shoot in the middle</td>
</tr>
<tr>
<td>Minimum number of leaves</td>
<td>50-100 leaf blades</td>
<td>100 petioles</td>
</tr>
<tr>
<td>Treatment of the leaves</td>
<td>Wash quickly in distilled water³ and then dry</td>
<td>Wash quickly in distilled water and then dry</td>
</tr>
</tbody>
</table>

¹ In relation to the size of the vineyard.
² The position according to the training system. The text of the Resolution includes illustrations of the correct sampling point for the major training systems in Europe.
³ Only for the determination of copper, zinc and manganese.

• In general, grapevines have a very low nitrogen requirement relative to most other crops. For example, 10 tons of grapes remove only about 25 lbs. of nitrogen from the vineyard.

• Growing grapes in soils previously used for vegetables, and thus, heavily fertilized, can result in excessive nitrogen contents of juice and EC levels in wine.

• The concentration of nitrogenous components such as arginine in juice and urea in wine increases proportionally with increasing nitrogen fertilization in the vineyard. If arginine concentrations in juice exceed 1000 mg/L, the vineyard must be considered over-fertilized.

• In a nitrogen deficient soil, an application of 100 lbs N/acre would achieve a level of about 150 mg of yeast available nitrogen/L of juice. This is considered sufficient for completion of fermentation. However, application of such high fertilizer rates can lead to excessive canopy growth and delayed fruit maturation.

Potential nitrogen deficiencies in juices in regard to yeast nutrition should not be addressed through vineyard fertilization.
VITICULTURE

2 Cover Crops

- Growers need to be aware that they may be adding a significant amount of nitrogen to their vineyards when they disk under winter legumes used as cover crops in the vineyard. The legumes include vetches (*Vicia* ssp.), clover (*Trifolium* ssp.), and pea (*Pisum* spp.). Use of these cover crops can increase vine nitrogen to excessively high levels. For example, clover tissue contains about 2.5% nitrogen, vetch tissue about 4%. Growing vetch can accumulate up to 75 lbs of nitrogen per acre, compared to an average of 25-50 lbs. per acre applied through commercial fertilizer additions. If legumes are used as cover crops, soil and vine nitrogen status should be monitored in order to avoid excessive arginine levels in juices. If additional nitrogen fertilization of the soil is to be avoided, plowing-under of winter legumes should not be practiced.

3 Cultivars & Rootstocks

- Different grape cultivars exhibit variations in nitrogen uptake, with some varieties being generally lower in level of arginine than others. However, low nitrogen status of cultivars is largely related to own-root characteristics and will change with the use of different rootstocks. Total bloom petiole nitrogen can vary by more than 40% on average, while nitrate-nitrogen may even vary 10-12 fold depending on the rootstock-scion combination used. Rootstocks can therefore have a profound impact on vineyard nitrogen status and fertilizer management. Local viticulture farm advisors can provide information on nitrogen uptake by different rootstocks.

RECOMMENDATION:

Growers should be aware of vine and grape nitrogen status and modify vineyard procedures if juice arginine concentrations are significantly greater than 1000 mg/L.
**Juice Nutrient Status/Additions**

- To correctly determine the nutritional status of an individual grape juice, it may be necessary to measure the level of nitrogen compounds actually available to the yeast for its metabolic activity. Nitrogen status of grapes varies widely with vineyard site, soil, irrigation and fertilization practices, vintage weather, scion and rootstock, and grape maturity. The two major sources for nitrogen in must are ammonia and amino acids with the exception of proline. Proline cannot be used as a yeast nitrogen source without molecular oxygen, which is not present in an anaerobic grape juice fermentation.

Both available nitrogen sources can be analyzed in a winery lab or at contract laboratories. Ammonia assays include enzymatic/photometric tests or use of an ammonia probe. Yeast available amino acids may be measured rapidly by an colorimetric OPA/NAC test (Dukes & Butzke 1996), which requires a spectrophotometer. Analysis by HPLC determines all nitrogen sources simultaneously but requires highly skilled personnel and yields delayed results under winemaking conditions. It is necessary to obtain analytical results within a few hours after yeast inoculation in order to make decisions about nutrient additions to deficient musts.

It would also be desirable to determine critical arginine levels through a simple analytical procedure which can be applied in a winery lab. There is currently no rapid analysis for arginine in juice available to wineries today. Development of a procedure has been proposed by UC Davis.

- To avoid sluggish or stuck fermentations, it is permitted by BATF to add up to 8 lbs of diammonium phosphate (DAP) per 1,000 gal (960 mg/L) to a nitrogen deficient must, which translates into ca. 200 mg of nitrogen/L. However, excessive levels of nitrogen may contribute to urea formation and excretion by yeast. Although some nutrients are required to accomplish an optional malolactic fermentation, high nutrient levels at the end of fermentation can contribute to microbial instability of a wine (see lactic acid bacteria).

- *Yeast food* preparations may add an unidentified level of yeast available nitrogen to a juice. It is recommend that winemakers request the supplier to specify the different nitrogen sources.

- The use of urea as a fermentation supplement is prohibited. BATF has found that the use of urea is not considered acceptable in good commercial practice among wine producers and has rescinded the listing of urea as an authorized treatment (Federal Register, Vol. 55, No. 118, 24974-24982, 06/19/90).

**Recommendation:**

Winemakers should know the nitrogen status of their juices and not over-supplement with diammonium phosphate.
## Yeast Strains

- Wine yeast strains differ in their ability to rapidly catabolize urea during fermentation. When excess urea accumulates in the cell’s cytoplasm, it is released into its environment, the must. High urea producing yeasts are those that have a high capacity to degrade arginine to urea, but a low urea metabolizing ability. Low urea metabolizing ability may result from low activity of urea amidolyase, inhibition of amidolyase activity by the presence of high levels of ammonia, deficiencies of cofactors required by amidolyase, or apparently low activity due to hyperactive arginase. Genetic as well as environmental factors influence the amount of urea released by the cells. Some commercial yeast strains such as Lallemand 71B®, Red Star SC1120® and Premier Cuvée (PdM)® have been described as producing relatively low levels of urea. Yeast companies will be able to recommend their lowest urea excreting strains for each specific winemaking application, and it is suggested that they be consulted.

- Spontaneous fermentations with undefined yeast strains will necessitate monitoring of arginine in juices and urea and EC levels in every fermentation. It is not clear what impact natural fermentations will have on EC levels as this has not been thoroughly investigated. However, it is anticipated that indigenous yeast strains will display a similar variability in urea catabolism as observed in commercial strains.

**RECOMMENDATION:**

If juice is high in arginine content, fermentation should be inoculated with known low-urea producing strains of yeast appropriate for the winemaking application.

## Lactic Acid Bacteria

- Certain wine lactic acid bacteria are capable of forming small amounts of citrulline, a precursor of ethyl carbamate, from the amino acid arginine, and excreting this precursor into the wine. Routine nitrogen supplement of juices with unknown nutritional status can increase the potential for bacteria available nitrogen status for arginine. Additionally, even strains not able to degrade arginine may produce small increases in ethyl carbamate, suggesting that nitrogenous precursors other than those derived from arginine may be involved. Research results indicate the need for caution in the selection of starter cultures for conducting malolactic fermentation in wine, since citrulline formation from arginine degradation could result in elevated levels of ethyl carbamate, even at normal temperatures, during prolonged storage. In addition, spontaneous malolactic fermentation by undefined strains should be avoided, as this may lead to formation of ethyl carbamate precursors.

**RECOMMENDATION:**

If malolactic fermentation is desired, winemakers should either use a commercial strain that does not produce high levels of citrulline or monitor juice for citrulline content post-fermentation.
Ureases

- Since urea is the major precursor for EC in wine, enzymatic hydrolysis of urea to ammonia and CO₂ appears to be a suitable way to eliminate formation from this source. Preparations of urease enzyme are commercially available and permitted by BATF for the treatment of wine. However, urease activity is severely limited under normal wine conditions, specifically with respect to low pH and ethanol. Urease is especially inhibited by high concentrations of malic acid, and fluoride residues (from cryolite® application in the vineyard) in excess of 1 mg/L. Any combination of these factors make it practically impossible to reach the desired low urea levels in reasonable time, even at a very high enzyme dosage. A complete elimination of EC is not possible.

**RECOMMENDATION:**

If wine is high in residual urea, winemakers may be able to use a urease treatment to reduce urea levels. However, the effectiveness of the urease addition must be evaluated for each wine to confirm the enzyme is active.

Sur lie aging

- It is a common winemaking style to age wine *sur lie* (on the yeast lees) after primary fermentation in order to impact the wine’s organoleptic properties. Aging on the lees leads to the liberation of nitrogenous compounds, amino acids and protein, into wine: a rapid excretion from the intracellular pool of the yeast cells during the first weeks of storage, and a slow increase during further storage due to autolysis of the yeast.

- However, it has been reported that, in wines made from grapes of low amino acid concentration, after extended lees contact, no increase in ethyl carbamate concentration was found, and that no additional ethyl carbamate precursors were released from the yeast during extended lees contact. Therefore, under above conditions, the practice of extended yeast lees contact appears not to raise ethyl carbamate potentials.

- No data are available to document an influence of sur lie aging on urea concentrations of wine made from grapes with excessive concentrations of yeast assimilable nitrogen.

- Similarly, no data are available regarding the production of sparkling wine and the evolution of levels of urea and other EC precursors during yeast autolysis, e.g. during long-term aging of tirage-bottled wines.

**RECOMMENDATION:**

*Sur lie* aging has not been shown to dramatically impact ethyl carbamate levels, but this has not been thoroughly tested.
DISTILLATION/FORTIFICATION

• Although urea is not volatile and EC itself possesses a poor volatility, EC may still be found in wine distillates. It can be formed post-distillation via the reaction of a volatile precursor, isocyanate, and ethanol both at ambient and elevated temperatures.

• Producers of fruit wine distillates have to be aware of another precursor for ethyl carbamate, in the form of cyanides. Stone-fruits, especially such as cherries, apricots or plums, contain sugar-bound cyanides in their seeds, which can be released during fermentation. Removal of stones prior to fermentation, and a secondary distillation are essential to avoid high concentrations of volatile EC precursors in this type of spirit.

• Producers of fortified wines have to take the same considerations into account as table winemakers, since fortification itself may aggravate the problem of urea excretion by yeast. Urea is often formed during the early and middle stages of fermentation with subsequent yeast generations utilizing it during the later stages. Maximum excretion occurs frequently, but with exceptions, at about 12° to 16° Brix. Arresting fermentation at this stage will lead to high urea concentrations in the fortified wine. It is recommended to perform a test fortification in the winery lab and to analyze both the fermenting wine and the resulting dessert wine for urea.

• In addition, the fortifying grape spirit or brandy can serve as the primary source for EC taint in fortified wines, and should be monitored for EC and potential EC.

RECOMMENDATION:

Since isocyanate is formed by break-down of urea, the same recommendation as for table wine production applies. No recommendation can be given at this point regarding the fractionation of distillates due to lack of data regarding the distillation behavior of volatile EC precursors.

SHIPMENT AND STORAGE

• The chemical reaction between urea and ethanol increases exponentially with temperature. It is therefore essential that a wine containing elevated levels of urea is not exposed to elevated temperatures (above 100°F) during storage or shipment.

RECOMMENDATION:

Since long-term exposure of wine to heat is also detrimental to its sensory properties and visual stability, wineries should educate and encourage the shipper, distributor, wholesaler, and retailer to minimize heat exposure by use of appropriate insulated containers, shipping schedules and storage facilities.
SUMMARY

Avoid excessive nitrogen fertilization in the vineyard.

Monitor soil nitrogen status.

Monitor vine nitrogen status.

Do not use winter legumes as cover crops if soil nitrogen status is already high.

Be aware that nitrogen uptake varies strongly with different cultivars and especially rootstocks.

Monitor juice nitrogen status.

Do not add excessive nitrogen supplements.

Do not add nitrogen supplements routinely.

Do not add urea as nitrogen supplement.

Avoid juice arginine levels greater than 1000 mg/L.

When choosing among wine yeast strains, avoid those with high urea excretion characteristics.

Use malo-lactic bacteria with known characteristics.

Be aware that use of urease preparations cannot completely eliminate EC formation.

Be aware that must fortification may aggravate the problem of urea excretion by yeast.

Monitor EC levels of fortification spirit.

Avoid exposure of wine to elevated temperatures during storage and transport.
BIBLIOGRAPHY

• Almy J; Ough C., 1989
  Urea Analysis For Wines.
  Journal Of Agricultural And Food Chemistry, V37 N4:968-970.

• An D; Ough C., 1993
  Urea Excretion And Uptake By Wine Yeasts As Affected By Various Factors.

• Ari’izumi-K; Suzuki-Y; Kato-I; Yagi-Y; Otsuka-K; Sato-M, 1994
  Winemaking From Koshu Variety By The Sur Lie Method: Changes In The Content Of Nitrogen Compounds.
  American Journal of Enology and Viticulture, V45 N3, 312-318

• Bell, S.J., 1994
  Ph.D. Thesis, School of Agriculture, University of Western Australia

• Bisson, L. F. 1996
  Ethyl Carbamate.

• Boulton, R.B. et al., 1995
  Ethyl Carbamate.
  In: Principles and Practices of Winemaking, Chapman & Hall, New York, 166-167

• Boulton, R.B., 1992
  The Formation of Ethyl Carbamate from Isocyanate and Ethanol at Elevated Temperatures.
  In: Elaboration et Connaissance des Spiritueux, BNIC, Cognac, France, 339-343

• Christensen, L.P., Luvisi, D. and Schrader, P., 1996
  The Effect Of Rootstock On Nutrient Uptake.
  American Vineyard, 5(4), 14 16

• Daudt C.; Ough C.; Stevens D; Herraiz T., 1992
  Investigations Into Ethyl Carbamate, N-Propyl Carbamate, And Urea In Fortified Wines.
  American Journal Of Enology And Viticulture, V43 N4:318-322.

• Dukes, B.; Butzke C., 1996
  Concentration of α-Amino Compounds in Grape Juice can be Rapidly Determined Using an o-Phthalaldehyde/N-acetyl-L-cysteine Spectrophotometric Assay.
  47th Annual Meeting of the American Society for Enology & Viticulture, Reno, NV.

• Famuyiwa O.; Ough C., 1991
  Modification Of Acid Urease Activity By Fluoride Ions And Malic Acid In Wines.
  American Journal Of Enology And Viticulture, V42 N1:79-80.

• Faulh C; Wittkowski R., 1992
  Determination Of Ethyl Carbamate In Wine By GC-SIM-MS After Continuous Extraction With Diethyl Ether.
• Federal Register, 1990a
  FDA: Urethane in Alcoholic Beverages; Research and Survey Reports, Availability
  Vol. 55, No. 5 10816-10817, 03/23/90

• Federal Register, 1990b
  BATF: Revision and Recodification of Wine Regulations
  Vol. 55, No. 118 24974-24982, 06/19/90

• Fujinawa S.; Kodama S.; Todoroki H.; Suzuki T., 1992
  Trace Urea Determination In Red Wine And Its Degradation Rate By Acid Urease.

• Fujinawa S.; Todoroki H.; Ohashi N.; Toda J., 1990
  Application Of An Acid Urease To Wine - Determination Of Trace Urea In Wine.
  Journal Of Food Science, V55 N4:1018+.

• Henschke P.; Ough C., 1991
  Urea Accumulation In Fermenting Grape Juice.

• Herraz-T; Huang-Z; Ough-CS, 1993
  Amino Acids And Ethyl Esters Of Amino Acids In Sparkling And ‘sur Lie’ Wines.
  Italian Journal Of Food Science, V5 N1, 11-20

• Huang Z; Ough C., 1989
  Effect Of Vineyard Locations, Varieties, And Rootstocks On The Juice Amino Acid Composition Of Several Cultivars.
  American Journal Of Enology And Viticulture, V40 N2:135-139.

• Huang Z; Ough C., 1991
  Amino Acid Profiles Of Commercial Grape Juices And Wines.

• Huang Zx; Ough C., 1993,
  Identification Of N-Carbamyl Amino Acids In Wines And In Yeast Cells.
  American Journal Of Enology And Viticulture V44 N1:49-55.

• Karumanchiri, A., 1996
  Ethylcarbamate: From its Discovery in 1979 to its Current Status, Based on Test Data of 16,000 Wines.
  47th Annual Meeting of the American Society for Enology & Viticulture, Reno, NV.

• Kodama S., 1996
  Optimal Conditions For Effective Use Of Acid Urease In Wine.
  Journal Of Food Science, V61 N3:548-552.

• Kodama S.; Suzuki T., 1995
  Highly Sensitive Method For Urea Detection In Wine.
  Journal Of Food Science, V60 N5:1097+.

  Arginine Catabolism In Wine Lactic Acid Bacteria - Is It Via The Arginine Deiminase Pathway Or The Arginase-Urease Pathway.
  Journal Of Applied Bacteriology, V81 N5:486-492.
• Liu, S.Q. et al., 1995. Occurrence Of Arginine Deiminase Pathway Enzymes In Arginine Catabolism By Wine Lactic Acid Bacteria. Applied and Environmental Microbiology. 61: 310-316.


• Mauricio J.; Millan M.; Moreno J; Ortega J., 1995 Changes In The Urea Concentration During Controlled Wine Aging By Two Flor Veil-Forming Yeasts. Biotechnology Letters, V17 N4:401-406.


• Monteiro F.; Bisson L., 1992 Utilization Of Arginine By Yeast During Grape Juice Fermentation And Investigation Of The Possible Role Of Arginine As A Precursor Of Urea. American Journal Of Enology And Viticulture, V43 N1:18-22.


• Ough C., Stevens D, Almy J., 1989 Preliminary Comments On Effects Of Grape Vineyard Nitrogen Fertilization On The Subsequent Ethyl Carbamate Formation In Wines. American Journal of Enology and Viticulture, 40(3) 219-220


• Ough C.; Stevens D; Sendovski T; Huang Z; And Others., 1990 Factors Contributing To Urea Formation In Commercially Fermented Wines. American Journal Of Enology And Viticulture, V41 N1:68-73.


• Spayd, S.E., Nagel, C.W., Edwards, C.G., 1995
  Yeast Growth In Riesling Juice As Affected By Vineyard Nitrogen Fertilization.
  American Journal of Enology and Viticulture, 46(1), 49 55.

  American Journal of Enology and Viticulture, 45(1), 34 42

  American Journal of Enology and Viticulture, 44(4), 378 386

• Stevens D.F., 1995
  Ethyl Carbamate Formation in Wines Undergoing Long-Term Storage.
  M.S. Thesis, Dept. of Viticulture & Enology, University of California, Davis.

• Stevens D.F.; Ough C., 1993
  Ethyl Carbamate Formation - Reaction Of Urea And Citrulline With Ethanol In Wine Under Low To Normal Temperature Conditions.

• Stoewsand G.; Anderson J.; Munson L., 1991
  Inhibition By Wine Of Tumorogenesis Induced By Ethyl Carbamate (Urethane) In Mice.
  Food And Chemical Toxicology, V29 N5:291-295.

• Tegmolarsson I.; Henick-Kling T., 1990
  The Effect Of Fermentation And Extended Lee Contact On Ethyl Carbamate Formation In New-York Wine.

• Tegmolarsson I.; Spittler T., 1990
  Temperature And Light Effects On Ethyl Carbamate Formation In Wine During Storage.
  Journal Of Food Science V55 N4:1166+.

• Trioli G; Ough C., 1989
  Causes For Inhibition Of An Acid Urease From Lactobacillus Fermentum.

• Webster, D.R., Edwards, C.G., Spayd, S.E., Peterson, J.C., Seymour, B.J., 1993
  American Journal of Enology and Viticulture, 44(3), 275 284.

• Zoecklein, B.W. et al., 1995
  Ethyl Carbamate.