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Glass Distilling Collector Applied for HCN Recovery from Submerged Culture Broth and Fruiting Body of *Pleurotus eryngii* for Identification and Quantification

PEI-YU CHOU,† CHIAN-HUEI HONG,† WENLUNG CHEN,‡ YU-JANG LI,‡ YEN-SHANG CHEN,§ AND ROBIN Y.-Y. CHIOU*,†

Department of Food Science and Department of Applied Chemistry, National Chiayi University, Chiayi, Taiwan, and Glory Biotechnology Company, Chiayi, Taiwan

Detection and surveillance of food commodities containing cyanide is a crucial issue of food safety. In this study, five strains of *Pleurotus eryngii* (*P. eryngii*) were grown in submerged culture of yeast malt broth (YMB) with the suspected production of HCN. A safety-warranted U-bent glass distilling collector with three enlarged bulbs on each arm was designed to recover the broth vapor. When $AgNO_3$ solution was used as an absorbent to interact with the vapor, a white precipitate was formed. The precipitate was isolated and identified as AgCN by FT-Raman spectroscopic analysis. When the absorbent was substituted by KOH, after evaporation to dryness, dissolved in D_2O, and followed by ^{13}C-NMR analysis, a KCN spectrum was achieved. Formation of AgCN and KCN confirmed HCN production in the broth by *P. eryngii*. When a sodium picrate solution (1.4%) was used as an absorbent and various authentic KCN solutions were applied for distillation and followed by absorbance determination at 510 nm, a linear dose-dependent relationship was obtained and the procedure was applied for HCN quantification of the marketed *P. eryngii* mushrooms (fruiting body). As estimated, 67.3% of the products contained HCN less than 1.0 mg/kg, 17.3% between 1.0 and 2.0 mg/kg, and 15.4% higher than 2.0 mg/kg. When the mushrooms were sliced and cooked in water at 95 °C for 6 min, 89.1% of the original HCN was lost. When the *P. eryngii* strains were respectively grown by submerged cultivation in YMB or YMB supplemented with 2.5% glycine for 16 days, HCN content was slightly higher in the latter than in the former for each strain.

KEYWORDS: HCN; mushroom; *Pleurotus eryngii*; picric acid; C^{13}-NMR; FT-Raman; submerged cultivation

INTRODUCTION

HCN is a highly toxic chemical in nature and mostly produced by plants (*1*) and microorganisms (*2*, *3*). Some food commodities such as cassava, sorghum, and mushrooms also contain HCN (*1*, *4*−*9*). HCN mainly interrupts electron transfer and ATP production and results in consequent cell death. The acute lethal dose of HCN was 0.5−3.5 mg/kg for humans, 3.7 mg/kg for mice, 0.5−10.0 mg/kg for rats, 2.0 mg/kg for cattle, and 1.5 mg/kg for dogs (*1*). As estimated during inhalation of HCN in air for a man, LD_{50} is 200 ppm for 30 min and 600−700 ppm for 5 min (*10*). Based on the nature of HCN being very toxic, volatile, and mildly smelly, prevention of inhalation of HCN by the laboratory personnel is equally important as prevention of oral intake to provide safety warranty. Various methods have been validated for HCN quantification in the literature (*4*, *6*, *7*, *11*−*16*). Generally, cyanide can occur in a natural matrix as free cyanides (HCN, CN^-), weak cyanides (metal complexed cyanide) and strong complexed cyanides (bound to cell constituents). Based on the reported methods, a further improved procedure by acidification and boiling distillation to determine the quantity of all forms of cyanide is needed.

In our laboratory, we initially did not expect the presence of HCN in the submerged culture of *Pleurotus eryngii* (*P. eryngii*) when the broth was subjected to purification of tyrosinase inhibitor. Tyrosinase inhibitory activity of the broth disappeared after vacuum concentrating, and the active fraction did not have absorbance at 254 nm monitored by HPLC fractionation. In addition, the active HPLC fraction was poisonous to a small fish (peacock fish) and heat stable in a screw-capped tube when subjected to heating in boiling water for 10 min. Thus, a toxic volatile in the broth was suspected. As referred to the literature, 1.1−26 ppm of HCN has been detected in the commercial *P. eryngii* mushrooms in Japan (*17*). HCN was also detected in the commercial *P. eryngii* mushrooms in Germany and Switzerland (*8*). So far, HCN production has not been reported in the submerged culture of *P. eryngii*. In this study, a U-bent glass distilling collector was designed with three enlarged bulbs on

* To whom all correspondence should be addressed. Fax: +8865-2775524. E-mail: rychiou@mail.ncyu.edu.tw.
† Department of Food Science, National Chiayi Unversity.
‡ Department of Applied Chemistry, National Chiayi Unversity.
§ Glory Biotechnology Co.

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each arm to facilitate HCN recovery for identification and provide personnel safety. The method was further applied for determination of HCN contents of the locally marketed *P. eryngii* mushrooms and their residual HCN contents when subjected to hot water cooking. During submerged cultivation, the determination of HCN production as affected by supplementation of glycine with yeast−malt broth (YMB; Difco Co., Detroit, MI) was extended.

MATERIALS AND METHODS

Strains and Fruiting Bodies of *Pleurotus eryngii*. Two culture collected strains of *Pleurotus eryngii* designated as BCRC 36037 and BCRC 36163 were obtained from Culture Collection Center, Food Industrial Research and Development Institute, Hsin-Chu, Taiwan. For tissue cultivation from a fruiting body of *P. eryngii*, a stalk taken from a freshly harvested fruiting body was surface sterilized with 1.0% NaOCl for 1 min and followed with 75% ethanol for 2 min. After three times of rinsing with sterile water, the stalk was aseptically cut into cubes (ca. 0.2 × 0.2 × 0.2 cm^3). A cube was placed onto a yeast−malt agar (YMA; Difco Co., Detroit, MI) plate and incubated at 28 °C for about 10 days, and a colony with ca. 6 cm diameter was formed in each dish. From the colony edge, a square agar plaque (0.4 × 0.4 cm^2) was removed and subcultured onto a new YMA plate for further cultivation. After about 10 days, a colony with ca. 6 cm diameter in each dish was stored in a refrigerator (4−5 °C) for culture collection. Originated from different mushroom sources, three isolated strains designated as RCPE-1, RCPE-2, and RCPE-3 were used in this study.

For commercial *P. eryngii* fruiting body sampling, mushrooms were collected from local markets and mushroom farms at varied times and locations. Fresh mushrooms were stored in a refrigerator (4−5 °C) and subjected to HCN determination within a week.

Mycelial Starter Preparation and Submerged Cultivation of *Pleurotus eryngii*. For wheat grain starter preparation, 50 g of wheat grains were soaked with water for 4 h and deposited into a 500 mL flask for autoclaving. Then, from an YMA plate grown with *P. eryngii*, five small square agar plaques (ca. 0.4 × 0.4 cm^2) containing hyphae were aseptically removed, inoculated, and incubated at 28 °C for 10 days, and the grains were covered with a mycelial mat. The flasks were stored at 4 °C for further use. In preparation of mycelial starter, approximately 50−80 grains were taken from the flask and deposited into a sterile 300 mL flask containing 100 mL of deionized water. The flask was shaken vigorously to release the mat to prepare a mycelial suspension for inoculation.

For submerged cultivation, a series of 500 mL flasks containing 180 mL of YMB supplemented with 2.5% glycine were prepared, and 20 mL of the above mycelial suspension was inoculated. The flasks were cultivated in an orbital shaker bath at 26−28 °C and 150 rpm for 16 days. The cultivated broth after being filtered through a sintered glass sieve was subjected to HCN recovery and identification.

Designing a Distillation Apparatus To Recover Broth Vapor. After several prototypes have been tried and modified, a distillation apparatus containing a glass distilling "Robean" collector (rolled-peanut shape) (**Figure 1**) was designed with consideration for optimizing vapor recovery and providing for personnel safety when dealing with hazardous volatile chemicals. The collector was mainly composed of a U-bent glass tube with three swollen bulbs in each of the inlet and outlet arms. Through a silicone stopper, the collector was adapted onto a 500 mL flask. A paralleled thermometer was also inserted to indicate the internal temperature. The second inlet bulb and the last outlet bulb were further enlarged to prevent absorbent outflow. The level of the enlarged inlet bulb was lower than that of the outlet bulb to enlarge the contact surface and enhance the mixing effect and vapor recovery.

For distillation of the submerged cultivated broth of *P. eryngii*, 100 mL of broth and a stirring bar were deposited into the 500 mL flask and the Robean collector was filled with 1.0 mL of 0.5 M $AgNO_3$. The Robean collector was adapted immediately right after addition of 0.2 mL of phosphoric acid into the flask to acidify the broth. Then, the flask was stirred (ca. 20 rpm), heated to boiling, and held for 30 min. After distillation, approximately one-third of the first enlarged inlet bulb was occupied by the absorbent solution and the final recovered volume was between 8 and 9 mL. As resulted from spontaneous chemical reaction, a white precipitate was observed when $AgNO_3$ was used as an absorbent and the precipitate was suspected to be AgCN. The white precipitates were collected by centrifuging, washed twice with deionized water, dried, and subjected to analysis with FT-Raman spectroscopy. A standard AgCN with 99% purity (Acros Organics, NJ) as a reference was analyzed concurrently.

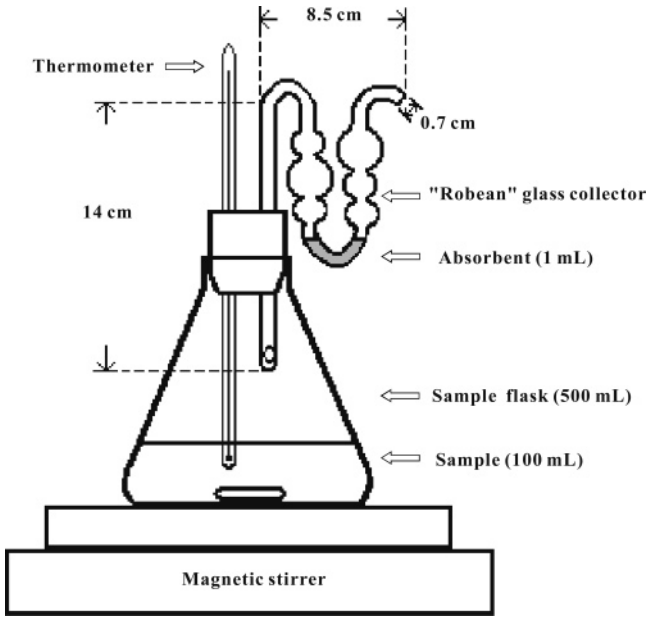

Figure 1. Schematic illustration of a distillation apparatus containing a "Robean" collector applied for HCN recovery.

For another chemical confirmation of the suspected volatile as HCN, the $AgNO_3$ solution was substituted with 0.5 N KOH for distillation. After 30 min of distillation of 100 mL of broth, the absorbent solution was deposited in a 250 mL flask and air-dried in a venting hood. After dissolving into 0.5 mL of D_2O, the solution was subjected to C^{13}-NMR analysis. A standard KCN (Merk, Darmstadt, Germany) as a reference was run concurrently.

AgCN and KCN Identification with RT-Raman and ^{13}C-NMR Spectroscopy. For analyzing FT-Raman spectra of the samples, a Bruker RFS-100 FT-spectrophotometer (Bruker Optik GmbH, Lubeck, Germany) was used. The sample (ca. 1 mg) was put into the tiny hole of a stainless steel holder for Raman measurement. Continuous-wave near-infrared excitation at 1064 nm was provided by a diode laser pumped Nd:YAG laser (Coherent Lubeck GmbH, Lubeck, Germany). The laser light with power of 150 mW was introduced and focused on the sample. The scattered radiation was collected at 180° with an ellipsoidal mirror and was filtered, modulated, and reflected back into the highly sensitive GaAs detector which was cooled by liquid nitrogen. Raman spectra were produced over the Raman shift of 0−3500 cm^{-1}. Typically, 500 interferograms were co-added at 4 cm^{-1} resolution with a sampling time of about 15 min.

For C^{13}-NMR analysis, the sample originally extracted from 100 mL of broth was dissolved in 0.5 mL of D_2O and subjected to NMR analysis using a Varian Mercury Plus 300 NMR spectrometer. An authentic KCN was run concurrently as a reference. For peak overlapping comparison of the spectra, the sample solution obtained from the broth of *P. eryngii* was spiked with an equal amount of KCN (ca. 0.1 mg dissolved in 0.5 mL of D_2O) and subjected to NMR analysis.

HCN Quantification with Picrate Solution. The procedures reported by Williams and Edwards (*13*) and Egan et al. (*16*) for HCN determination using picrate solution was modified. Using the designed apparatus (**Figure 1**), 1.0 mL of sodium picrate solution (1.4%, w/v, dissolved in 2.5% Na_2CO_3) as absorbent was deposited into the glass Robean collector. After deposition of a sample solution (100 mL) and a magnetic stirrer into a 500 mL flask, the Robean collector filled with 1.0 mL of absorbent was immediately adapted after addition of 0.2

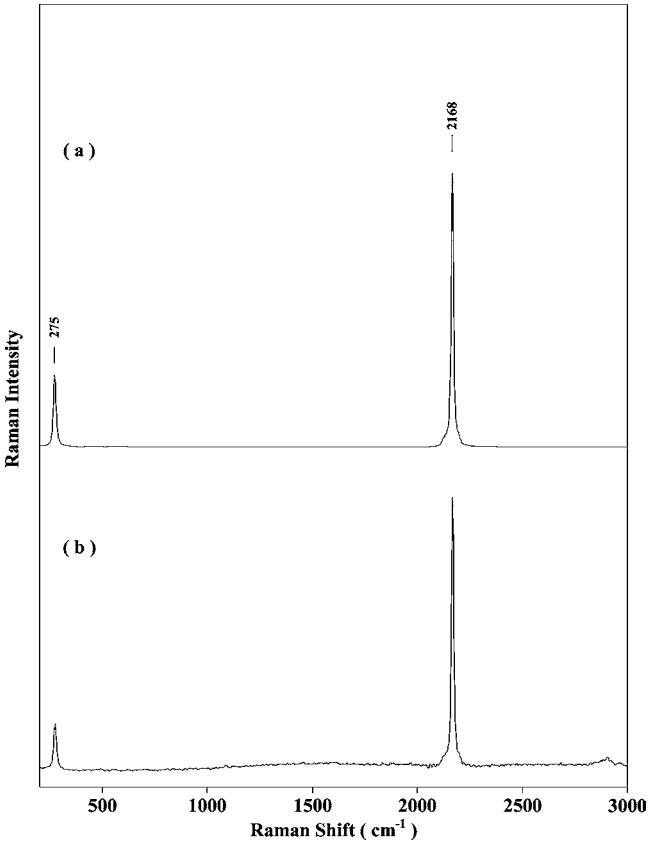

Figure 2. Raman spectra of AgCN and white precipitate obtained by distillation of the submerged-cultivated broth of *Pleurotus eryngii* after reaction of the distillate with $AgNO_3$: **a**, AgCN; **b**, white precipitate.

mL of phosphoric acid to acidify the solution. The flask was placed onto a magnetic stirrer hot plate, stirred (ca. 20 rpm), heated to boiling, and held for 30 min for distillation and HCN collection in the Robean tube. Then, the absorbent (in general 8−9 mL of volume) in the Robean tube was withdrawn in a graduate cylinder and replenished with deionized water to 10 mL. From the cylinder 2 mL was taken, deposited into a test tube, heated at 80 °C with a water bath for 20 min, and followed by absorbance determination at 510 nm. When an orange-red or even brick-red color was observed (indicating high HCN concentration), the 10 mL absorbent was diluted with an appropriate volume of 0.14% picrate solution prior to absorbance determination.

For construction of standard curves applied for HCN quantification, a 4000 ppm KCN aqueous solution was prepared in a brown glass vial. By using the distillation apparatus, a series of 500 mL flasks respectively containing 100 mL of deionized water were added with 0.2 mL of phosphoric acid. Then, 0, 1, 5, 15, and 30 μL of the 4000 ppm KCN solution were respectively added and immediately adapted with a Robean collector containing picrate solution. The resulting concentrations for the solutions were 0, 0.04, 0.2, 0.6, and 1.2 ppm of KCN, respectively. After distillation, the absorbent was withdrawn and diluted with deionized water to 10 mL, from which 2 mL of the solution was respectively taken and heated at 80 °C for 20 min prior to absorbance determination at 510 nm. Linear regression of the KCN concentration versus the absorbance unit was conducted for coefficiency determination. HCN concentrations were estimated according to the obtained linear equation and converted from KCN to HCN based on molecular weight ratio.

For direct reaction of KCN with picrate to skip off distillation, 1.0 mL of 1.4% picrate solution was respectively deposited into six test tubes. Then, 0, 1, 5, 15, and 30 μL of the 4000 ppm KCN solution were added and followed by dilution with deionized water to 10 mL, from which 2 mL of the solution was respectively taken for absorbance determination at 510 nm, linear regression of KCN concentration versus absorbance, and estimation of HCN concentration following the procedure described above.

HCN Determination of the Fruiting Body of *Pleurotus eryngii*. In total 52 mushroom products collected from different times and locations of the local markets and mushroom farms were analyzed. From each fruiting body after being chopped into fine pieces, 20 g was sampled, weighed, and deposited into a 500 mL flask and replenished with 100 mL of deionized water. Then the flask was subjected to homogenization for 2 min at 8000 rpm (Polytron PT 3000, Kinematica AG, Littau, Switzerland). Right after addition of 0.2 mL of phosphoric acid into the flask, the flask was adapted with a Robean collector containing 1.0 mL of picrate solution (1.4%) and subjected to distillation and HCN determination in the manner described above.

Residual HCN of Fruiting Body of *Pleurotus eryngii* Subjected to Heating. From a series of freshly harvested fruiting bodies of *P. eryngii*, each was cut into slices. The slices originating from a fruiting body were randomly sampled, trimmed, and weighed to 20 g. Then, the slices were subjected to water heating at 95 °C for 0, 2, 6, and 20 min, respectively. After cooking, each slice was chopped into fine pieces, deposited into a 500 mL flask, replenished with 100 mL of deionized water, and followed by homogenization for 2 min (Polytron, 8000 rpm). After addition of 0.2 mL of phosphoric acid and immediately being adapted with a Robean collector containing 1.0 mL of 1.4% picrate solution, the flask was applied for distillation and HCN determination following the procedure described above.

HCN Production As Affected by Glycine Supplementation and Determination of Bacteria in the Cultivated Broth. Five strains of *P. eryngii* including BCRC 36037, BCRC 36163, RCPE-1, RCPE-2, and RCPE-3 were respectively applied for submerged cultivation in YMB and YMB supplemented with 2.5% glycine (Sigma Chemical Co., St. Louis, MO). A preliminary experiment showed that HCN production increased with an increase of glycine up to 2.5% of supplementation. The cultures were cultivated at 28 °C and 150 rpm. From the cultivated broth, 10 mL of the broth was taken periodically and replenished with 90 mL of deionized water and subjected to HCN determination using the glass distilling apparatus following the procedure described above. As a supplemental experiment, 0.1 mL of each submerged-cultivated broth after 16 days of incubation was aseptically withdrawn, spread onto a Tryptic soy agar (TSA; Difco) plate, and incubated at 28 or 37 °C for 48 h; formation of any contaminant bacterial colony was examined and enumerated.

RESULTS AND DISCUSSION

When a collector filled with an absorbent was designed to recover volatiles by distillation, expansion of the contact surface and extension of retention (reaction) time was the key point in enhancement of recovery efficiency. Because distillation was conducted at an elevated temperature, prevention of outflow or backflow of the absorbent from the collector resulting from interior vessel-pressure fluctuation was critical. In this study, several prototypes of distilling apparatus have been designed, constructed, tested, and modified before a satisfactory glass distilling "Robean" collector (**Figure 1**) was achieved. The apparatus was successfully applied for HCN recovery from the submerged culture of *P. eryngii* by acidification and boiling distillation. The collector was mainly composed of a U-bent glass tube with three swollen bulbs in each of the inlet and outlet arms. A thermometer was also inserted parallel with the Robean collector through a silicone stopper as an adaptor in connection with a 500 mL flask. The second inlet bulb and the last outlet bulb were further enlarged to prevent outflow or back-flow of absorbent during distillation. The level of the enlarged inlet bulb was slightly lower than that of the outlet bulb to provide a sufficient condensing surface and serve as a reservoir to buffer back-flow of the absorbent during distillation. It also resulted in a vigorous mixing effect to enhance HCN reaction with absorbent. In addition, the bulbs also provided enlarged room for condensing. For instance, after 30 min of distillation, the final volume of absorbent was between 8 and 9 mL and its inlet

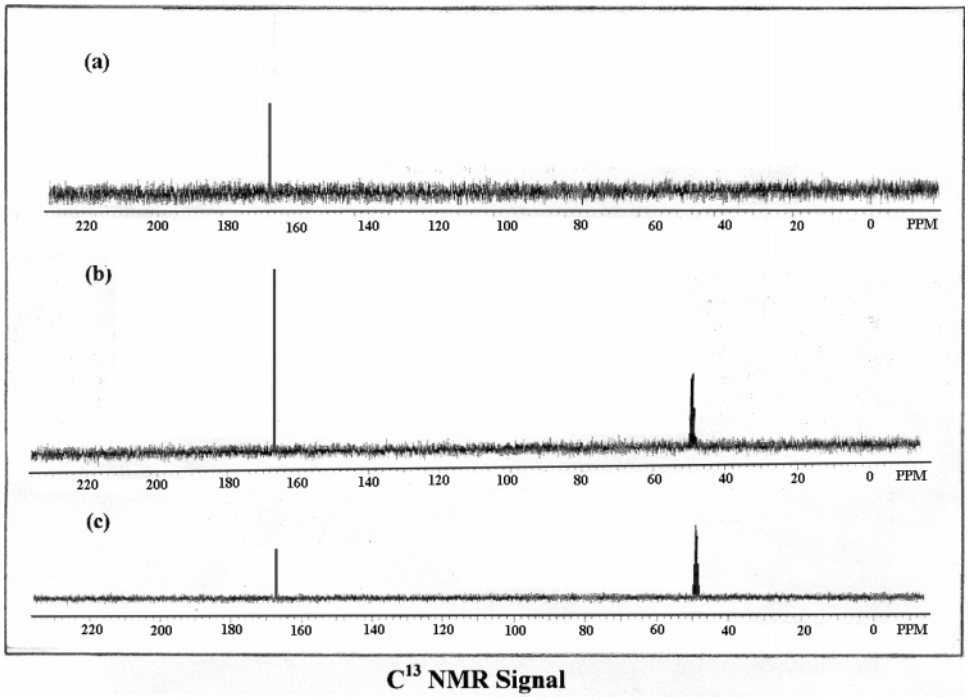

Figure 3. ^{13}C-NMR spectra of authentic KCN, purified crystal from *Pleurotus eryngii*, and the crystal mixed with KCN: **a**, authentic KCN; **b**, purified crystal of *P. eryngii*; **c**, purified crystal of *P. eryngii* and spiked with authentic KCN.

level was located at about one-third the bottom position of the enlarged inlet bulb.

When 1.0 mL of 0.5 M $AgNO_3$ was used as absorbent to recover distilled vapor from 100 mL of submerged-cultivated broth of *P. eryngii*, fine white precipitates were formed spontaneously. The white precipitates were isolated and identified as AgCN on the basis of performing an identical FT-Raman spectrum as that of authentic AgCN (**Figure 2**). When the intensity was magnified 40-fold to ensure its noise-free spectral quality, two peaks at 275 and 2168 cm^{-1} were obtained from the white precipitates which produced a typical vibrational mode of AgCN.

When $AgNO_3$ was substituted by 1 mL of KOH (0.5 N) as absorbent for distillation of the 100 mL broth of *P. eryngii*, after being evaporated to dryness, dissolved in D_2O, and followed by ^{13}C-NMR analysis, its spectrum (**Figure 3b**) was identical to that of authentic KCN (**Figure 3a**) having a signal at around 166 ppm (*18*). For further confirmation, when the sample was spiked with authentic KCN, the two integrated signals at around 166 ppm were completely overlapped (**Figure 3c**). On the basis of the FT-Raman and ^{13}C-NMR spectra, it is sufficient to confirm HCN production in the submerged culture of *P. eryngii*.

Picric acid is sensitive enough to react with HCN to form color for absorbance determination at 510 nm (*12*, *13*, *16*). In this study, on the basis of the nature of HCN that it is volatile and could be released under an acidic condition, KCN was applied as a surrogate to construct a standard curve. For comparison, various concentrations of KCN including 0, 0.04, 0.2, 0.6, and 1.2 ppm were respectively subjected to direct reaction with picrate solution or indirect reaction with picrate solution using the glass distilling apparatus (**Figure 1**). After linear regression of the relationships between KCN concentration and absorbance resulted from direct and indirect reactions with picrate solution, the linear equations were $y = 0.7257x + 0.0633$ for the indirect method and $y = 0.6719x + 0.0771$ for the direct method, respectively. The coefficiency (R^2) was 0.9985 for the former and 0.9994 for the latter. On the basis of the fact that slopes for the two lines were very close, HCN loss or insufficient evaporation during distillation using the designed glass distillation apparatus could be ignored. The linear relationship and equation enabled the estimation of HCN concentration accordingly after conversion from KCN to HCN by molecular weight difference.

Table 1. HCN Contents of Fresh *Pleurotus eryngii* Mushrooms Collected from the Local Markets or Mushroom Farms

HCN concn (mg/(kg of fresh wt))	no. of sample (distribution (%))	av concn[a] (mg/(kg of fresh wt))
<1.0	35 (67.3)	0.36 ± 0.29
1.0–2.0	9 (17.3)	1.39 ± 0.31
>2.0	8 (15.4)	2.57 ± 0.25

[a] Mean of determinations (n = 35, 9, and 8 from top to bottom) and standard deviation.

When 52 commercial mushrooms (fruiting body) of *P. eryngii* were subjected to HCN determination, all samples contained HCN (**Table 1**). HCN concentrations for most products (67.3%) were less than 1.0 mg/kg, 17.3% between 1.0 and 2.0 mg/kg, and 15.4% higher than 2.0 mg/kg. In Japan, 1.1−26 mg/kg of HCN has been detected in the commercial *P. eryngii* mushrooms (*17*). In Germany and Switzerland, HCN was also detected in the commercial *P. eryngii* mushrooms (*8*). It is obvious that various amounts of HCN existed in *P. eryngii* fruit bodies around Asia and Europe. Since mushrooms could be contaminated by HCN-producing bacteria in nature (*19*), the HCN detected in the fruit body of *P. eryngii* might originate from the contaminated bacteria. HCN produced by some *Pseudomonas* species have been reported (*2*, *20*, *21*). Thus, it is necessary to confirm the capability of *P. eryngii* in the production of HCN. As a supplemental experiment conducted in this study, 0.1 mL of the submerged-cultivated broth was aseptically withdrawn, spread onto a TSA plate, and incubated at 28 or 37 °C for 48 h and consequently no bacterial colonies were observed (data not shown). This indicates that there was no bacterial contaminant in the broth.

Table 2. Changes of HCN Content of Sliced *Pleurotus eryngii* Mushrooms As Affected by Cooking ($n = 3$)

heating time (min)	relative HCN content[a] (%)
0	100
2	27.2 ± 1.3
6	10.9 ± 0.6
20	5.8 ± 0.8
cooking broth	ND

[a] Mean of determinations ($n = 3$) with standard deviation; ND, not detectable.

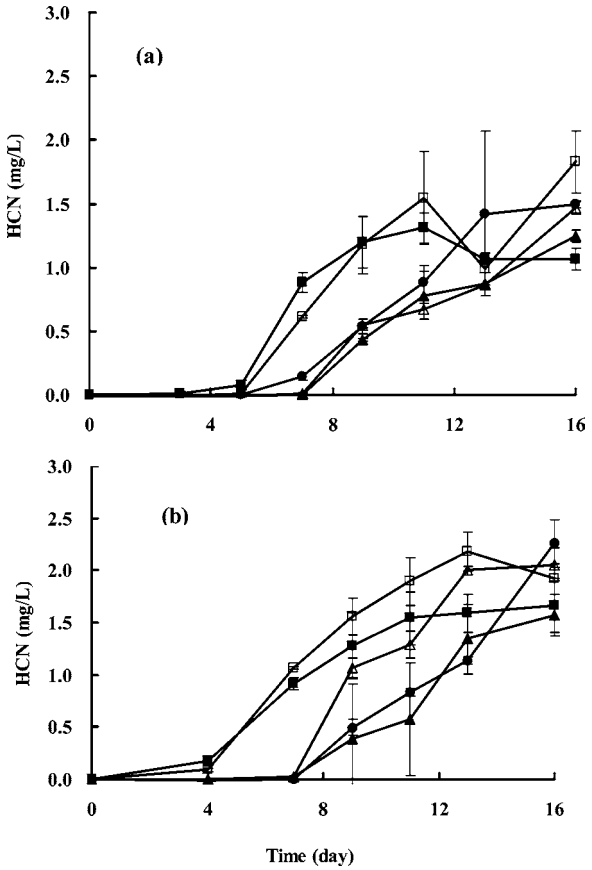

Figure 4. Changes of broth HCN contents during submerged cultivation of five *Pleurotus eryngii* with yeast malt broth (YMB) or YMB supplemented with 2.5% glycine ($n = 3$): **a**, YMB; **b**, YMB supplemented with 2.5% glycine. Symbols: ■, RCPE-1; ▲, RCPE-2; ●, RCPE-3; □, BCRC 36037; △, BCRC 36163.

The presence of HCN in the edible mushrooms is of public concern from the viewpoint of food safety. When the fruiting bodies were cut into slices and cooked in a water bath at 95 °C for 0, 2, 6, and 20 min, the residual HCN content decreased sharply during cooking (**Table 2**). The retained HCN after 2, 6, and 20 min of cooking were 27.2 ± 1.3, 10.9 ± 0.6, and 5.8 ± 0.8%, respectively. In Japan, when the fruit bodies of *Tricholoma giganteum* were grill roasted or cooked in boiling water, the residual HCN was 65% after 6 min of grill roasting of the whole fruit body and was 27% after 3 min of cooking the sliced fruiting body in boiling water (*17*). Therefore, consumption of the cooked edible mushrooms rather than raw ones is strongly recommended for food safety consideration.

When five strains of *P. eryngii* (two obtained from the Culture Collection Center and three isolated from fruiting bodies) were submerged-cultivated with YMB for 16 days and HCN contents were periodically determined (**Figure 4**), there was no HCN detected in the initial 3 days of cultivation. After 5 days of incubation, HCN contents increased gradually with increasing incubation time. Up to 11 days, the HCN concentrations changed in a limited range. After 16 days of incubation, HCN concentrations for the cultures were 1.82 mg/L for BCRC 36037, 1.49 mg/L for RCPE-3, 1.46 mg/L for BCRC 36163, 1.24 mg/L for RCPE-2, and 1.06 mg/L for RCPE-1. On the basis of the fact that there was no bacterium detected when the broth was spread onto a plate count agar described above, it is the first time HCN production by *P. eryngii* has been detected in the submerged culture.

Glycine could be used by *Pseudomonas* species as a substrate to produce CO_2 and HCN under aerobic condition (*20*, *21*). In this study, when the five cultures of *P. eryngii* were cultivated in YMB or YMB supplemented with 2.5% glycine for 16 days, HCN concentrations for all cultures increased with an increase of cultivation time (**Figure 4**). For each culture, the HCN concentration was slightly higher in the latter than the former. The most significant difference of HCN production was detected for the strain of RCPE-3. The average HCN contents were 1.49 ± 0.04 mg/L for RCPE-3-grown in YMB and 2.26 ± 0.3 mg/L for it grown in YMB supplemented with 2.5% glycine. It is apparent that during submerged cultivation of *P. eryngii* HCN production might be enhanced by supplementation of glycine in the medium.

In summary, the Robean collector designed in this study was unique from the conventional distillation equipments. By using various absorbents with the collector, such as $AgNO_3$, KOH, and sodium picrate solution, it is available to recover HCN from the submerged culture of *P. eryngii* for identification and accurate quantification of HCN released from the commercial *P. eryngii* mushrooms. Extensive applications of the distillation apparatus for other purposes in volatiles characterization are of worthy further investigations.

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