High-Performance Liquid Chromatographic Determination of ε-Pyrrole-lysine in Processed Food

Grace H. Chiang

ε-Pyrrole-lysine—a glucose–lysine Amadori compound—has been identified in the water-soluble fraction of many processed foods by an isocratic HPLC method. The method employed a C8 reversed-phase column, a 0.5% NaOAc buffer (pH 4.3) mobile phase, and an electrochemical (EC) detector. ε-Pyrrole-lysine was found to be highly electroactive. With EC detection, as little as 0.5 ppm of ε-pyrrole-lysine was detected. The method was applied to various storage samples such as nondairy creamer, dry dog food, instant gravy, powdered meal replacer, dry milk, hot cocoa mix, snack bars, and semimoist sauce. The measured ε-pyrrole-lysine level in each product reflected shelf life and storage conditions closely. Moreover, ε-pyrrole-lysine level maintained its upward trend even under severe heat treatment (at 110 °C) and suggested that it may serve as an indicator of the "advanced" Maillard reaction.

The lysine–glucose reaction system is considered as a model of the Maillard reaction between reducing sugar and the ε-amino group of lysine (Nakayama et al., 1980; Kato et al., 1982; Lee et al., 1984; Warmbier et al., 1976). Besides the brown pigments (melanoidins), compounds identified in lysine–glucose reactions system are monofructosyllysine, difructosyllysine, HMF, furans, pyrroles, and pyridines (Finot et al., 1968; Feather and Harris, 1973; Olsson et al., 1981; Kato et al., 1982; Lee et al., 1984). In searching for simple and rapid analytical tools to objectively express the extent of nonenzymatic browning Maillard reaction, I had previously reported a HPLC method to determine furosine (Chiang, 1983). Furosine is an acid hydrolysis product of ε-deoxyfructosyllysine. ε-Deoxyfructosyllysine is the condensation and rearrangement product between the carbonyl group of reducing sugar and the ε-amino group of lysine. This Amadori-type compound (1-amino-1-deoxy-2-ketose) formation is involved in the first stage of the Maillard reaction (Nursten, 1981). As the Maillard reaction advances, the Amadori compounds are converted into aldehydes and dicarbonyls via dehydration, fission, and Strecker degradation. The carbonyl compounds are then reacted with other available amines and form melanoidins (high molecular weight brown substances) eventually. Hurrell and Carpenter (1981) called reactions leading to the formation of Amadori compounds "early" Maillard rections. Reactions after which are "advanced" Maillard reactions. The furosine method had been demonstrated to be a good indicator of shelf life and storage conditions in numerous products. Since ε-deoxyfructosyllysine is a product during early Maillard reactions, it decomposes as the reactions progressed into advanced state. Accordingly, a drop in furosine level was observed in samples with long shelf storage. The furosine method alone thus cannot be used to express the extent of Maillard reaction especially on long-term stored products.

ε-Pyrrole-lysine [2-amino-6-[2-formyl-5-(hydroxymethyl)pyrrol-1-yl]hexanoic acid] was first identified and synthesized by Nakayama et al. (1980). The authors later (Kato et al., 1982) monitored its presence in the water-soluble fraction of a reaction mixture of glucose and lysine by TLC. The chemical structure of ε-pyrrole-lysine suggested it is an advanced Maillard reaction product (through private communication with P. A. Finot). It is the objective of this study to establish a simple and sensitive analytical method to determine ε-pyrrole-lysine in real food systems and to explore the possibility of using ε-pyrrole-lysine as an indicator for advanced Maillard reactions.

Research and Development, Calreco, Inc., Van Nuys, California 91412.

EXPERIMENTAL SECTION

Reagents. ε-Pyrrole-lysine was synthesized according to Nakayama et al. (1980). The synthesized ε-pyrrole-lysine was identified and confirmed by MS analysis (performed by Analytical Service group at American Hospital Supply Corp.). HPLC–UV analysis revealed that the ε-pyrrole-lysine was >90% pure. The absolute amount of ε-pyrrole-lysine in the synthesized ε-pyrrole-lysine was calculated using its UV absorbance at 297 nm and the extinction coefficient reported by Nakayama et al. (1980).

The mobile phase used for HPLC separation consisted of 0.5% acetate buffer prepared by dissolving sodium acetate in Millipore-filtered water and adjusting the solution to pH 4.3 with glacial acetic acid. Test samples were obtained from product development, packaging, and product stability groups at Calreco, Inc.

Apparatus and Operating Conditions. The HPLC setup consisted of a Spectraphysics (Model 8700) solvent delivery system, a Waters WISP autosampler, and an ESA EC detector (Model 5100A). Signals from the detector were monitored by a Perkin-Elmer LCI-100 microprocessor. HPLC was performed at ambient temperature. Separations were performed either on a E. Merck Lichrosorb RP8-Cartridge column (4.0 mm (i.d.) × 250 mm, 7 μm) or on an Altex Ultrasphere Octyl column (4.6 mm (i.d.) × 250 mm, 5 μm). The mobile phase was eluted isocratically through the column at a flow rate of 1.6 mL/min. For EC detection, the guard cell voltage was set at 0.7 V and only one of the two ESA's parallel dual cells was activated; the applied voltage was set at 0.55 V.

Sample Preparation. *Dry Milk, Powdered Meal Replacer, Hot Cocoa Mix, and Instant Gravy.* A total of 1 g of sample was weighed out in a 20-mL screw-cap vial. To the sample was added 8 mL of warm water (60–65 °C), and the contents were vortex-mixed for 1 min. The sample solution was then deproteinized with the addition of 1 mL of $ZnSO_4$ (33.4 g/100 mL) and 1 mL of $K_4Fe(CN)6{\cdot}3H_2O$ (17.29 g/100 mL). The solution was then centrifuged at 24730*g* (or 10000 rpm) for 15 min on a Sorvall centrifuge (Model RC-5C, SM-24 rotor). After Millipore filtering, the supernatant was analyzed by HPLC.

Snack Bars and Instant Refried Beans. A total of 2 g of sample was weighed out in a Virtis homogenizer cup. Of a 1% solution of sulfosalicyclic acid 20 mL was added. The sample mixture was then homogenized with the Virtis homogenizer at medium-high speed for 2 × 1 min. The

0021-8561/88/1436-0506$01.50/0

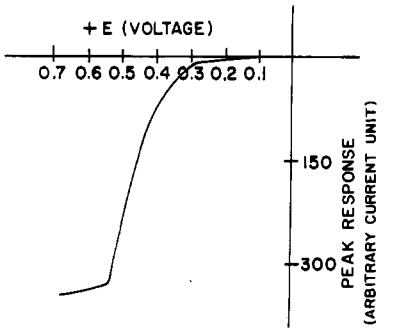

Figure 1. Current–voltage (C–V) curve of ϵ-pyrrole-lysine. Oxidation of ϵ-pyrrole-lysine at ESAs porous graphite coulometric electrode. HPLC conditions: Lichrosorb RP8 cartridge column; mobile phase, 0.5% acetate buffer, pH 4.3; flow rate, 1.6 mL/min.

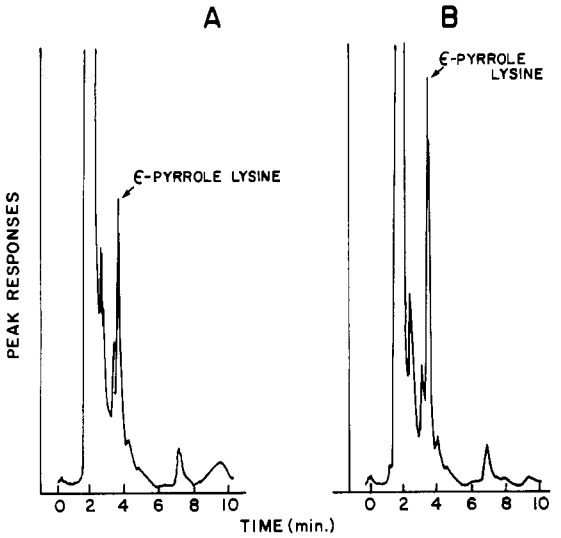

Figure 2. HPLC chromatograms of nondairy creamers: (A) 8 weeks of storage at 22.2 °C; (B) 8 weeks of storage at 45.0 °C. Chromatographic conditions: Lichrosorb RP8 cartridge column; mobile phase, 0.5% acetate buffer, pH 4.3; flow rate, 1.6 mL/min.

sample solution was centrifuged at 24730g on a Sorvall centrifuge (Model RC-5C, SM-24 rotor), and after Millipore filtering, the supernatant was analyzed by HPLC.

Nondairy Creamer. A glass wool plugged small funnel was placed on top of a 20-mL screw-cap vial. A total of 1 g of sample was weighed out and placed on the funnel. To the sample was added 3 × 4 mL of $CHCl_3$. With each addition, the $CHCl_3$ was allowed to drain slowly into the vial and was then discarded. The sample was air-dried to get rid of remaining $CHCl_3$. Warm water (2 × 4 mL; 60–65 °C) was then added to the sample. A disposable pipet was used to push the glass wool at the top of the funnel down to the vial, and the same pipet was used to rise out remainder sample on the funnel. The sample solution was then deproteinized with addition of 1 mL of $ZnSO_4$ (33.4 g/100 mL) and 1 mL of $K_4Fe(CN)_6{\cdot}3H_2O$ (17.29 g/100 mL). The funnel was then removed, and the vial was capped. After vortex mixing, the solution was centrifuged under the same conditions as applied on other types of samples. The supernatant was Millipore-filtered and followed by HPLC analysis.

RESULTS AND DISCUSSION

The C–V (current–voltage) curve obtained by repeatedly injecting standard ϵ-pyrrole-lysine (0.92 μg/mL) into the HPLC and by varying the applied voltages of the EC detector is shown in Figure 1. As shown, peak responses reached the maximum at an applied voltage of 0.55 V. ϵ-Pyrrole-lysine is a highly electroactive compound. A

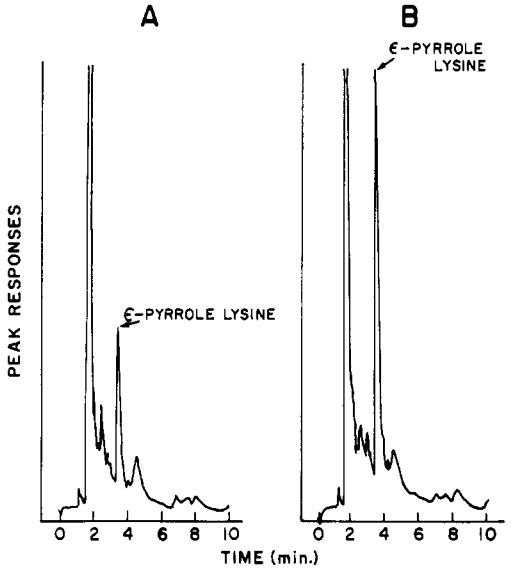

Figure 3. HPLC chromatograms of snack bars: (A) 18 weeks of storage at 29.4 °C; (B) 18 weeks of storage at 37.8 °C. Chromatographic conditions: Lichrosorb RP8 cartridge column; mobile phase, 0.5% acetate buffer, pH 4.3; flow rate, 1.6 mL/min.

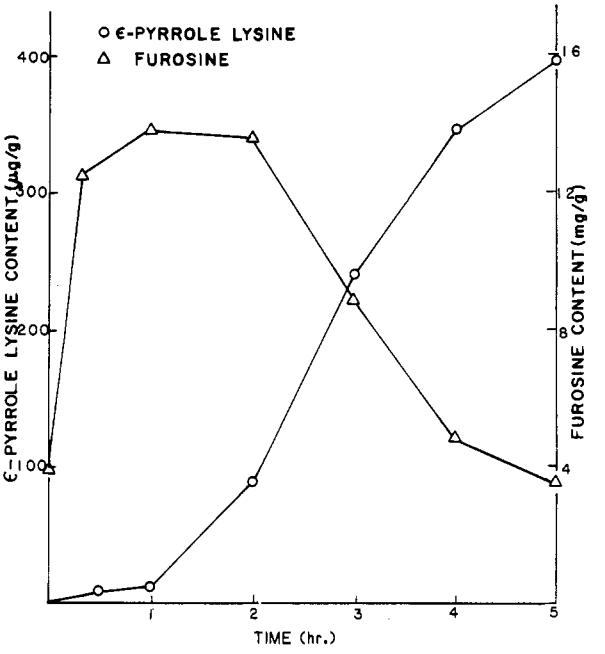

Figure 4. ϵ-Pyrrole-lysine and furosine contents of powdered meal replacer (vanilla flavor) under experimental conditions (heated at 110 °C for up to 5 h).

complex C–V curve is expected of ϵ-pyrrole-lysine because its electroactivity was believed to be due to the aldehyde functional group and the unstable heterocyclic structure. A simple sigmoidal C–V curve is obtained. The EC detection of ϵ-pyrrole-lysine provided high selectivity and sensitivity. Although ϵ-pyrrole-lysine is a UV chromophore, the existence of other UV chromophores in the test samples made UV detection less selective than EC detection. It was found that the sensitivity of the assay was increased by 10-fold as off-column eluent was monitored by EC rather than UV (laboratory observation).

A number of processed food products, both under experimental conditions and under normal storage conditions, were analyzed by the HPLC-EC method. Figures 2 and 3 show comparisons of control and storage samples of nondairy creamer and snack bar. Table I lists ϵ-pyrrole-lysine contents in samples under experimental (ac-

Table I. ε-Pyrrole-lysine in Samples at Experimental Conditions

sample	time, h (80 °C)	no. of determin	ε-pyrrole-lysine level, ppm found in sample	spike level	rec, %
powdered meal replacer	0	2	1.49 ± 0.06		
	1	2	3.54 ± 0.10		
	2	2	5.04 ± 0.04		
	4	3	9.14 ± 0.15	20.3	93.39
	6	2	13.12 ± 0.04		
nonfat dry milk	0	2	1.96 ± 0.00		
	1	2	6.25 ± 0.16		
	2	5	20.32 ± 0.01 (day 1)	19.74	91.87
			20.77 ± 0.33 (day 2)		
	4	2	75.21 ± 0.54		
	6	2	133.06 ± 4.27		
hot cocoa mix	0	2	6.82 ± 0.12		
	1	2	7.21 ± 0.06		
	2	3	10.80 ± 0.27	19.68	95.86
	4	2	12.81 ± 0.05		
	6	2	16.44 ± 0.19		
cheese	0	3	12.73 ± 0.27	70.63	105.33
gravy	1	2	46.23 ± 1.15		
	2	2	76.68 ± 0.57		
	4	2	152.63 ± 1.75		
	6	2	227.68 ± 13.27		
nondairy creamer	0	2	1.37 ± 0.06		
	1	2	7.87 ± 0.45		
	2	2	16.72 ± 0.88		
	4	2	31.42 ± 1.10		
	6	2	44.75 ± 1.29		
dry dog food	0	2	2.50 ± 0.06		
	1	2	5.67 ± 0.68		
	2	2	24.37 ± 2.47		

Table II. ε-Pyrrole-lysine in Samples under Calreco Storage Conditions

sample	storage conditions: temp, °C (no. weeks)	no. of determin	ε-pyrrole-lysine level, ppm found in sample	spike level	rec, %
nondairy creamer 1	45.0 (8)	4	18.97 ± 0.92 (day 1)		
			18.59 ± 0.31 (day 2)		
	22.2 (8)	2	8.72 ± 0.15		
nondairy creamer 2	37.8 (132)	4	24.63 ± 0.98 (day 1)		
			23.41 ± 0.33 (day 2)		
	4.0 (132)	2	0.92 ± 0.14		
formed meal bar	4.0 (5)	6	27.83 ± 0.71		
	37.8 (5)	2	39.31 ± 0.47	49.56	94.29
snack bar	37.8 (18)	2	71.85 ± 3.20		
	29.4 (18)	2	46.04 ± 0.59		
hot cocoa mix	22.2 (192)	2	3.38 ± 0.02		
	0.0 (192)	2	4.20 ± 0.05		
refried beans	−17.8 (27)	2	25.34 ± 0.11		89.25
	37.8 (27)	2	19.45 ± 0.08		
tomato pasta sauce	45.0 (2)	3	9.08 (day 1)	18.70	92.96
			9.21 ± 0.06 (day 2)		
	4.0 (2)	2	26.31 ± 0.23	20.08	99.24

celerated) conditions, and Table II lists ε-pyrrole-lysine contents in Calreco storage samples. Tables I and II contain results of replicate preparations and results from preparations on different days. As shown, reproducibility of the method was satisfactory. The average percent deviation among replicate preparations is 2.85, and the average percent deviation between day to day preparations is 1.89%. Various samples were also spiked with standard ε-pyrrole-lysine, and at least 90% of the spiked ε-pyrrole-lysine was recovered after sample processing (Tables I and II). No detection limit problem was encountered with the EC detection throughout the study. Detection limit varies with sample. Judging from nearby background interferences and ε-pyrrole-lysine peak responses, ε-pyrrole-lysine concentrations of as low as 0.5 ppm should be detectable.

As indicated from the results, ε-pyrrole-lysine level corresponds with storage conditions closely. Levels of ε-pyrrole-lysine increase with time and temperature for most dry products except refried beans. The trend was reversed in semimoist sauce and refried bean samples. It is believed that ε-pyrrole-lysine is a intermediate product during glucose–lysine reactions. It degradates as storage conditions become more severe. That explains why ε-pyrrole-lysine decreases with time and temperature under the reactive liquid semimoist state. Nevertheless, no reduction in ε-pyrrole-lysine levels was observed during the 5-h period at 80 °C on all tests products (Table I). In fact, ε-pyrrole-lysine seemed to be relatively stable as compared to furosine in dry systems. Figure 4 shows levels of ε-pyrrole-lysine and furosine found in powdered meal replacer products (vanilla flavor) during a 5-h period at 110 °C. As noticed, furosine level reached a maximum within 1 h and then started to decline. ε-Pyrrole-lysine level, on the other hand, was still increasing after 5 h. It then can be concluded that the ε-pyrrole-lysine level gives a better indication of storage conditions, especially long-term and elevated temperature, than furosine.

The HPLC ε-pyrrole-lysine method is simple and accurate. Typical sample analysis can be completed within 30

min. In dry systems, the ϵ-pyrrole-lysine level represented the extent of nonenzymatic Maillard reactions well. Thus, the method not only is suited for shelf life quality control purposes but also is valuable for elucidating Maillard reaction mechanisms.

Registry No. ϵ-Pyrrole-lysine, 74509-14-1.

LITERATURE CITED

Chiang, G. H. "A Simple and Rapid High-Performance Liquid Chromatographic Procedures for Determination of Furosine, Lysine Reducing Sugar Derivative". *J. Agric. Food Chem.* **1983**, *31*, 1373–1374.

Feather, M. S.; Harris, J. F. "Dehydration Reactions of Carbohydrates". *Adv. Carbohydr. Chem. Biochem.* **1973**, *28*, 161–224.

Finot, P. A.; Bricont, J.; Viani, R.; Mauron, J. "Identification of a New Lysine Derivative Obtained Upon Acid Hydrolysis of Heated Milk". *Experientia* **1968**, *24*, 1097–1099.

Hurrell, R. F.; Carpenter, K. J. "The Estimation of Available Lysine in Foodstuffs after Maillard Reactions". *Prog. Food Nutr. Sci.* **1981**, *5*, 159–176.

Kato, H.; Nakayama, T.; Sugimoto, S.; Hayase, F. "Volatile and *Nonvolatile* Maillard Reaction Products between L-Lysine and D-Glucose". *Agric. Biol. Chem.* **1982**, *46*, 2599–2600.

Lee, C. M.; Sherr, B.; Koh, Y.-N. "Evaluation of Kinetic Parameters for a Glucose–Lysine Maillard Reaction". *J. Agric. Food Chem.* **1984**, *32*, 379–382.

Nakayama, T.; Hayase, F.; Kato, H. "Formation of (2-Formyl-5-hydroxymethylpyrrol-l-yl)-L-norleucine in the Maillard Reaction between D-Glucose and L-Lysine". *Agric. Biol. Chem.* **1980**, *44*, 1201–1202.

Nursten, H. E. "Recent Developments in Studies of the Maillard Reaction". *Food Chem.* **1980**, *6*, 263–277.

Olsson, K.; Pernemalm, P. A.; Theander, O. "Reaction Products and Mechanism in Some Simple Model Systems". *Prog. Food Nutr. Sci.* **1981**, *5*, 47–55.

Warmbier, H. C.; Schnickels, R. A.; Labuza, T. P. "Nonenzymatic Browning Kinetics in an Intermediate Moisture Model System: Effect of Glucose to Lysine Ratio". *J. Food Sci.* **1976**, *41*, 981–983.

Received for review February 5, 1987. Accepted September 4, 1987.

Terpenoid Aldehydes in Upland Cottons: Analysis by Aniline and HPLC Methods

Robert D. Stipanovic,* David W. Altman, Deborah L. Begin, Gerald A. Greenblatt, and John H. Benedict

Lysigenous pigment glands in Upland cotton contain a diverse mixture of terpenoid aldehydes, including gossypol, hemigossypolone, and heliocides H_1, H_2, H_3, and H_4. These terpenoids are involved in plant resistance to some phytophagous insects. A high-performance liquid chromatographic (HPLC) method has been developed for quantitating each of these terpenoids in seed, leaves, and flower buds using a data base from 14 cultivars and experimental lines grown in five diverse field environments with four replicates per environment. Hemigossypolone and heliocides H_1, H_2, H_3, and H_4 were the major terpenoid aldehydes in leaves, and gossypol was the major tepenoid aldehyde in flower buds and essentially the only one in seed. Results from these analyses have been compared with those obtained on the same tissue by the aniline method of analysis; aniline analysis detected an average of about 50% of the terpenoid aldehydes in leaves and flower buds compared to the HPLC method. Seed analysis by the two methods gave nearly identical amounts of gossypol. HPLC analysis provides the concentration of each individual terpenoid aldehyde, while the aniline method measures only the total terpenoid aldehydes. The former method gives a more accurate measure of total terpenoid aldehydes, yet because the aniline method is fast, relatively inexpensive, and highly correlated with terpenoid content in plant tissues as determined by the HPLC method (r = 0.80–0.99, $P < 0.01$), it remains a useful procedure.

Cotton (*Gossypium spp.*) and other members of the Gossypieae tribe contain lysigenous glands in vegetative and reproductive plant parts (Lukefahr and Fryxell, 1967). Cottonseed is a rich source of protein, but its use for human consumption or animal feed has been limited by a toxic substance, gossypol, in the seed glands. The structure of gossypol (G) was determined by Adams and co-workers (review 1960) as shown in Figure 1. On the basis of the assumption that G is the only terpenoid aldehyde in pigment glands, Smith developed a spectrophotometric method for determining G in seed (1958) and in leaves and flower buds (1967). Smith's method involved reaction of G with aniline to form a yellow Schiff base. With this method, both free and so-called "bound gossypol" could be determined (Smith, 1967). Bound refers to G that has the aldehyde group condensed with free amino groups on proteins to form a Schiff base. The formation of a Schiff base is a reversible reaction; when heated with excess aniline, bound gossypol is liberated to form the soluble aniline Schiff base. Free and bound gossypol taken together are called total gossypol. A modification of Smith's method is widely used by breeders and entomologists (Dilday, 1983). Pons et al. (1958) developed an alternative method for determining total G in cottonseed based on the aniline reaction using 3-amino-1-propanol as a complexing agent.

Studies on insect resistance to *Heliothis*, *Spodoptera*, and other insect pests assumed G was the terpenoid aldehyde conferring resistance [reviews by Bell and Stipa-

Southern Crops Research Laboratory, USDA—ARS, P.O. Drawer JF, College Station, Texas 77841 (R.D.S., D.W.A., D.L.B.), Biochemistry and Biophysics, Texas A&M University, College Station, Texas 77843 (G.A.G.), and Texas A&M University and Texas Agricultural Experiment Station, Corpus Christi, Texas 78410 (J.H.B.).

0021-8561/88/1436-0509$01.50/0