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Determination of lysinoalanine formed during alkali-treating and aging of Chinese preserved eggs by gas chromatography

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ABSTRACT

We established a method of gas chromatography with flame ionization detector (GC-FID) using N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) as derivatisation reagent and DL-2,6-diaminopimelic acid (DPA) as internal standard for the determination of lysinoalanine (LAL) in Chinese preserved egg. The derivatisation reaction was under the optimized condition of 75 °C and 30min. Method feasibility was demonstrated by experimental assessments of the limit of detection (32 mg kg^{-1} of LAL in protein) and the limit of quantitation (95 mg kg^{-1} of LAL in protein), linearity (r^2 = 0.9996), the relative standard deviation values (< 5%) and mean recovery (91.9%-94.0%). The contents of LAL in Chinese preserved egg white and yolk during pickling and aging period were measured. The data revealed a change rule that the LAL content in egg white and yolk was increased from 0 to 17078.62 mg kg^{-1} of protein and from 0 to 8434.21 mg kg^{-1} of protein during the alkaline pickling period, and then gradually raised to 17481.44 mg kg^{-1} and 8956.16 mg kg^{-1} of protein after 18 days of aging, respectively.

Keywords: Gas chromatography with flame ionization detector (GC-FID), lysinoalanine, Chinese preserved egg, change rule

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INTRODUCTION

A new cross-linking amino acid, N^{ε}-(DL-2-amino-carboxyethyl)-L-lysine (lysinoalanine) was first found in alkali (pH 13) treated ribonuclease A (RnaseA)[1]. Heat and/or alkaline or high pressure treatment can promote the formation of LAL in protein-containing foods [2,3]. LAL formation in foods results in lower digestibility of protein and bioavailability with a loss and racemization of essential amino acids [4-6]. In addition, LAL can inactivate metalloenzymes and cause unique lesions for rats, such as kidney damage, nephrocalcinosis or nephrocytomegaly [7-9]. So far, LAL has been found in a variety of foods or food ingredients, including cereal products, cooked meats, egg products, calcium (sodium) caseinate, infant formulas, pasteurized milk, etc [10-13].

Chinese preserved egg also known as Pidan, is a kind of delicious and nutritious traditional Chinese egg product. It also possesses the function of anti-inflammatory [14,15]. Now it becomes a popular food consumed in many countries, especially in Asia. It is generally made by pickling duck, chicken or quail eggs in alkaline, salt and metal ions mixtures solution at ambient temperature for 3-5 weeks [14-15]. During the pickling process, strong alkali may induce the formation of LAL in egg protein; however, few studies on LAL in Chinese preserved eggs have been reported.

Current research efforts have been motivated by the need to develop appropriate analytical procedures of evaluation

of LAL in a variety of foods. The techniques of amino acid analyzer and liquid chromatography (HPLC) have been extensively utilized for the LAL analysis [4,10,13,16-18]. The method of amino acid analyzer has an advantage of simultaneous analysis LAL and other amino acids, however, the instrument is expensive and the method shows large standard deviations due to interference of co-eluting compounds [16]. The HPLC methods for the determination of LAL with dansyl chloride (DNS-Cl) derivatisation need ensuring the appropriate dosage of DNS-Cl, otherwise many other structured co-eluting molecules may emerge and they will affect the accurate quantification [18]. When 9-fluorenylmethyl-chloroformate (FMOC-Cl) is used as derivatisation reagent with solid-phase extraction or combining with mass spectrometry (MS) in HPLC methods, the accuracy and sensitivity are higher, but the pre-processing of HPLC with SPE method is complex and time-consuming, and the cost of HPLC-MS is high [11,17]. Montilla et al.[11] and Bosch et al. [19] developed a GC-FID method for the quantitive detection of LAL in cheese, milk and some other foods with MTBSTFA derivatisation, which is simple and rapid for determining LAL without purification of samples. However, GC hasn't been evaluated for determining LAL in Chinese preserved egg. Although methods that combine GC and MS often provide high sensitivity and accuracy, the instruments are expensive and existing complex instrument maintenance problems, which is uneconomical for the determination of LAL in a large number of samples. Therefore, the aim of this study was to establish a GC-FID method to detect LAL in Chinese preserved egg. Basically, we investigated the change rule of LAL content in Chinese preserved egg white and yolk during alkali-treating and aging process.

EXPERIMENTAL SECTION

Chemicals and materials

LAL was obtained from Bachem (Bubendorf, switzerland). Hydrochloric acid (HCl), sodium hydroxide (NaOH), copper sulfate ($CuSO_4$), and sodium chloride (NaCl) of analytical grade were purchased from Sinopharm Chemical Reagent Co. Ltd (Shanghai, China). Ovalbumin, DPA, triethylamine (TEA), MTBSTFA, and N, N-dimethylformamide (DMF) were purchased from sigma-Aldrich (St. Louis, MO, USA). High purity water obtained from a Mill-Q purification system (Millipore, Bedford, MA, USA) was used to dilute concentrated hydrochloric acid.

Fresh duck eggs laid within 5 days (65-70 g) were purchased from a farm in Nanchang country, Jiangxi Province, China.

Preparation of preserved eggs

The preserved eggs were produced according to the previous method reported by Yang et al. [15] with some modification. Duck eggs were pickled in solution containing NaOH (4%, W/V), $CuSO_4$ (0.4%, W/V), NaCl (4%, W/V) at 25°C for 30 days. After 30 days of pickling, pickled eggs were cleaned with tap water, and then left at room temperature (15-25°C) for another 18 days for aging. Three eggs were collected each time at six-day intervals during the whole period. Egg white and yolk were separated manually. Both preserved eggs white and yolk samples were stored at -20°C until analysis.

Protein analysis

Protein content of Chinese preserved egg white and yolk were determined by Kjeldahl (N×6.25) in accordance with the standards by the International Diary Federation [20].

Acid hydrolysis

Chinese preserved eggs were minced and mixed with a homogenizer. Egg white, egg yolk and ovalbumin containing about 50 mg of protein were separately mixed with 10 mL of 6 M HCl in a pyrex tube, flushed with nitrogen to eliminate air, then sealed under vacuum pressure. These mixtures were hydrolysed at 110°C for 24 h. The hydrolysed samples were cooled at room temperature, and filtered through a 0.45 μm cellulose acetate filter.

Derivatisation

Derivatisation conditions exploration. The hydrolysed ovalbumin filtrate proved to be free of LAL was spiked with pure LAL to a concentration of 0.4 mM, and 500 μL of this solution was removed to a 1.5-mL vial, 200 μL of freshly prepared DPA reagent (0.37 mM in 0.01 M HCl) were added, and then blow-dried at 38°C with a Pressure Blowing Concentrator manufactured in Hangzhou, Zhejiang Province, China. Derivatisation of the dried samples was performed for variable MTBSTFA amount (40, 60, 80, 100 and 120 μL of pure MTBSTFA), various temperatures (55, 75, 95, 115 and 135°C) and different reaction times (15 , 30, 60, 90 and 120 mins). Derivatisation reaction was quenched by rapidly cooling to room temperature and the derivative mixtures were filtered through a 0.45 μm PTFE filter, then stored at 4°C until they were analysed by GC and the relative peak areas of the derivatives plotted as a function of silylation reagent dosage, temperature and time for establishing optimum derivatisation conditions.

Derivatisation of the standard and samples. The standard calibration solutions (0.05 mM, 0.1 mM, 0.2 mM, 0.4 mM, 0.8 mM, 1.6 mM) were prepared by stepwise diluting 3.2 mM LAL (in 0.01 M HCl)with acid-hydrolysed ovalbumin. 500 μL of different concentration of LAL or the filtered Chinese preserved eggs hydrolysate piked with 200 μL of 0.37 mM DPA solution, 90 μL of DMF and 10 μL of TEA were vigorously shaken for mixing. The mixtures were dried at 38°C with a Pressure Blowing Concentrator. Derivatisation reactions were under the optimum conditions, including adding suitable dosage of MTBSTFA at appropriate temperature for a suitable time for derivatisation. The filtered mixture was stored at 4°C until analysis.

GC-FID analysis

A Shimadzu GC-2014C equipped with a FID was used to analyse LAL in the samples. A HP-5 (30 m × 0.32 mm id, 0.25 μm film thickness) fused silica capillary column (Hewlett Packard, Avondale, PA, USA) was used for separation. Nitrogen at a flow-rate of 2 mL/min was used as the carrier gas. 1 μL of derivative mixtures was injected with a splitless mode. After an initial hold time of 1 min at 100°C, oven temperature was programmed to 250°C at a rate of 30°C /min and kept for 12.5 min, and then increased to 260°C at a rate of 5°C /min and held for 19.5 min. The injector and detector temperatures were 280°C and 300°C. Data were acquired by Shimadzu Chemstation system.

GC-MS/MS analysis

GC-MS/MS analysis was performed with an Agilent 6980N GC coupled with an Agilent 5973i quadrupole mass spectrometer (Palo Alto, CA, USA). The tBDMSi derivatives were separated on an Agilent HP-5MS (crosslinked 5% phenyl, 95% dimethylpolysiloxane) fused-silica capillary column (30 m × 0.32 mm × 0.25 μm film thickness). The carrier gas (helium) flow rate was 1 mL/min. Injections were operated in splitless mode and the injection volume was 1 μL. Injector and detector temperature were 280°C and 300°C, respectively. The oven program was as follows: hold for 1 min at 80 °C, ramp to 200 °C at 30 °C /min, ramp to 260 °C at 10 °C /min and hold for 25 min at 260 °C. The mass spectrometer was operated in the electron ionization mode (EI) at 70 eV. The retention times and the characteristic fragments of the EI mass spectra were determined using an Agilent Chemstation system by total ion monitoring.

Recovery, precision and accuracy

Relative standard deviation (RSD) and accuracy as percentage of relative error (CV, %) were determined by spiking the Chinese preserved egg white and yolk pickled for 30 days with the independent concentration of LAL. The acid hydrolysis, derivatisation and GC-FID analysis procedures were carried out according to the described above. Recovery ratio = (amount of LAL in spiked sample - amount of LAL in sample)/amount of LAL added ×100 %. The intra-day and inter-day precision were measured on the same day (n = 5) and over 3 consecutive days (n = 5).

Statistical analysis

Depending on the type of analysis, three or five replications were performed. Results were analysed by software origin 8.0. Statistical evaluation of data was conducted using one-way analysis of Duncan by SPSS 19.0 with a significance level of 95%.

RESULTS AND DISCUSSION

Qualitative analysis

MTBSTFA has been widely used to derivatise amino acids under mild condition [21-22]. Dimethyl-tert-butylsilyl (tBDMSi) derivatives produced by MTBSTFA are more stable to moisture, as well as hydrolysis, hydrogenolysis, mild reduction, and oxidation reactions than the corresponding TMS derivatives [21-22]. DPA has been reported to be a suitable internal standard for the analysis of LAL by GC [11]. Therefore MTBSTFA and DPA were separately used as derivatisation reagent and internal standard for monitoring LAL levels in Chinese preserved egg.

LAL peak was identified by migration time, standard addition and MS analysis. tBDMSi-DPA eluted at 19.0 min and tBDMSi-LAL showed a retention time of 31.6 min. The peak of tBDMSi-LAL was found in egg white and yolk of Chinese preserved egg soaked in alkaline solution for 30 days (Fig. 1a and b).The identification of LAL in Chinese preserved egg white and yolk samples were also performed by GC-MS/MS after derivatisation process. Although there was a little difference in the analysis condition between GC-MS and GC-FID, the LAL peak in GC-MS total ion chromatograph could be identified by comparing with that in GC-FID chromatograph. The mass spectrum of tBDMSi-LAL is shown in Fig. 2. According to the report of [11], tBDMSi-LAL could be characterized by the $[M-57]^+$ and $[M-15-131-159]^+$ ion (Fig. 2), which supported and confirmed the quantitative data of LAL as described above. Analogously, the tBDMSi-DPA peak identification could be strengthened by GC-MS/MS as well.

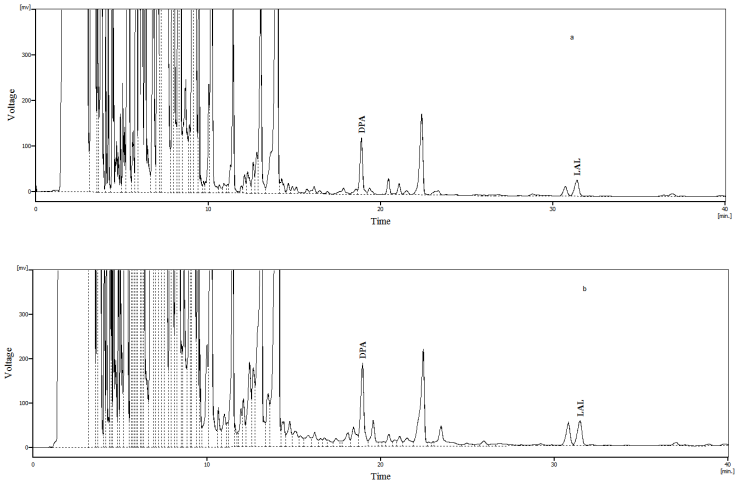

Fig.1 GC-FID profiles of tBDMSi amino acid derivatives of hydrolysed Chinese preserved egg white (a) and yolk (b) pickled for 30 days

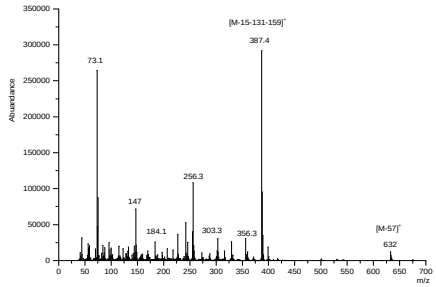

Fig.2 Mass spectrums of tBDMSi-LAL in Chinese preserved egg white/yolk soaked in alkaline solution for 30 days

Optimized derivatisation conditions

As Chinese preserved egg samples have complex matrix, and ovalbumin is the main protein of duck egg white. It was spiked with LAL standard to imitate the sample matrix to optimize derivatisation conditions. The objective of the investigation of derivatisation conditions was to achieve high tBDMSi derivatisation efficiency of LAL, which was evaluated by comparing the relative peak areas of tBDMSi-DPA and tBDMSi-LAL in different conditions. The influence of three parameters, such as the dosage of MTBSTFA, temperature, and time on tBDMSi derivatisation efficiency of LAL is marked in Fig. 3a, b and c, respectively. The larger relative peak area response of DPA and LAL means the lower derivatisation efficiency of LAL. The result shown in Fig. 3a indicated the derivatisation efficiency was the lowest, when 40 μL of MTBSTFA were added, and there was no significant difference (p> 0.05), when the consumption of MTBSTFA was 60 μL, 80 μL, 100 μL and 120 μL. Since other amino acids always participate in the competitive reaction during derivatisation process, 40 μL of MTBSTFA is not enough to derivatise LAL completely, and excess MTBSTFA contributed to maintaining the stability of tBDMSi-LAL [23]. The data in Fig. 3b indicated that no significant difference was found in the tBDMSi derivatisation efficiency of LAL at 75 °C and 95 °C, and it was higher at 75°C, 95 °C than that at 55°C and 135°C, because LAL derivatisation rate was relatively small at low temperature so that LAL couldn't be adequately derivatisated by MTBSTFA within a certain time, and LAL or tBDMSi-LAL might decompose as a result of the instability of LAL or tBDMSi-LAL at high temperature or in the presence of water. As can be observed in Fig. 3c, the derivatisation efficiency of LAL was increased when the derivatisation time was prolonged from 15 min to 30min, thereafter declined with time extension, and it was highest at 30 min (p< 0.05). This might be on account of the degradation of tBDMSi-LAL at constant temperature for a long time, and the existence of insufficient derivatisation in a short time. Hence we added 100 μL of MTBSTFA , chose 75 °C and 30min for derivatisation.

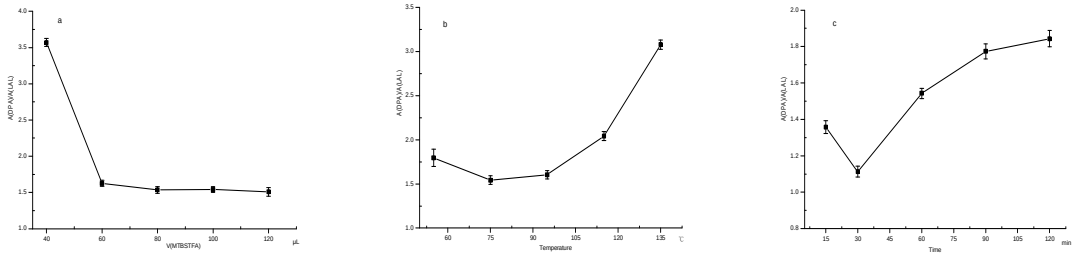

Fig.3 Effect of derivative reagent dosage (a), temperature (b) and time (c) on derivatisation efficiency of LAL with MTBSTFA

Method validation

Calibration curve. As six concentrations levels of LAL standard solution were separately mixed with DPA internal standard solution before they were derivatised by MTBSTFA, the final volumes could not be accurately calculated. But we kept maintaining the same volume of LAL standard solutions added DPA as hydrolysed Chinese preserved eggs samples spiked with DPA. Calibration curve equation (y = 8.643x- 1.123) was calculated with the ratio of DPA peak area/LAL peak area (y) and the quality ratio (x), which is intuitive and straightforward for LAL determination in samples. Good linearity was obtained for the range 1166-37333 mg kg^{-1} of LAL in protein, and the correlation coefficient was 0.9996. The results indicated the ion intensities of diluted LAL standard samples were not influenced by the presence of the internal standard.

Detection and quantitation limits. Limit of detection (LOD) and quantitation (LOQ) were respectively calculated on the basis of signal-to-noise ratios of 3:1 and 10:1 near the retention time of LAL. In this investigation, LOD and LOQ were 32 mg kg^{-1} and 95 mg $kg^{-1}$of LAL in protein. Therefore, Chinese preserved egg samples didn't need to be purified, because the selectivity of the method is high enough for analysing samples.

Analytical precision, repeatability, reproducibility and recovery. Table 1 shows the intra-day precision, mean recovery with the mean CV (%) by five replications of adding low, medium, high amount of LAL to Chinese preserved egg white and yolk. The mean recovery ranged from 92.2% to 94.0% with the mean CV (%) in the range of 5.9%- 7.8% in preserved egg white, and the mean recovery and CV (%) in preserved egg yolk were ranged 91.9%-92.6% and 7.3%-7.8%. the intra-day precision (RSD) for each samples was <5% and inter-day precision (RSD) was＞10%. The intra-day RSD reflects the reproducibility of the method is satisfying; however, the repeatability of a long time is relatively poor and unacceptable. Perhaps the inter-day instability is related to the degradation of tBDMSi-LAL for encountering moisture in the air or under a not excess enough of derivatisation reagent. For this reason, analysis runs must be made consecutively and completed within 24 hours of the start.

Table 1 Recovery, precision and accuracy of LAL spiked Chinese preserved eggs pickled for 30 days

samples	Spiked concentration (mg kg^{-1})	Mean recovery(%) (n=5)	intra-day RSD(%) (n=5)	Mean CV(%) (n=5)
Chinese Preserved egg white	819	92.3%	2.1	7.6
	1024	94.0%	2.5	5.9
	1229	92.2%	2.4	7.8
Chinese Preserved egg yolk	437	92.6%	1.6	7.3
	547	91.9%	4.0	7.6
	657	92.2%	1.7	7.8

Quantitative analysis and change rule of LAL in Chinese preserved egg during processing

During 30 days of alkaline-pickling and 18 days of aging period, the contents of LAL in Chinese preserved egg white and yolk were analysed. As LAL derives from protein, the final results were expressed in milligram per kilogram of protein and drew as a line chart. Fig. 4 shows the effect of alkali-pickling time on LAL formation in Chinese preserved egg white (Fig. 4a) and yolk (Fig. 4b). No detectable level of LAL was found either in fresh duck egg white or yolk, while the amount of LAL was 17078.62 mg kg^{-1} of protein (2047.91 mg kg^{-1} of fresh egg white) and 8434.21 mg kg^{-1} of protein (1094.88 mg kg^{-1} of fresh yolk), respectively in Chinese preserved egg white and yolk pickled for 30 days. And the LAL content in egg white and yolk were 17481.44 mg $kg^{-1}$of protein (2096.03 mg kg^{-1} of egg white) and 8956.16 mg kg^{-1} of protein (1162.86 mg kg^{-1} of yolk) after storing at room for 18 days. In the whole process, the trend of LAL content change in Chinese preserved egg white and yolk was slightly different from that in the yolk (Fig. 4a and b). LAL content in Chinese preserved egg white was significantly increased within 12 days of pickling ($p< 0.05$), then kept slightly elevated during 12-30 days of alkaline treating ($p> 0.05$), while LAL in yolk was significantly raised in quantity within the whole period of pickling ($p< 0.05$). The level of LAL both in preserved egg white and yolk gradually increased during aging process ($p> 0.05$). In addition, the content of LAL in egg white is always more than that in yolk during the same period. The result was correlated with the factors

affecting LAL formation, including source of protein, pH or alkaline concentration, heating temperature, treating time and with or without inhibitors [2,4,24]. The higher pH (alkaline concentration), the higher temperature or the longer exposure time, the more LAL can be formed unless the condition is too severe [2]. The different types and content of protein in egg white and yolk may be one of main reasons for different changes of LAL content between them. Egg white usually contains about 9.7%-10.6% of protein, which consists of ovalbumin, ovotransferrin, ovomucoid and others; however, the level of protein in the yolk fraction is 15.7%-16.6%, including low-density lipoprotein, livetin, phosvitin, high-density lipoprotein, etc [25]. Additionally, pH is also one of important inducing factors. OH^- continuously penetrated from the eggshell directly into the egg white, afterwards gradually into the egg yolk during the pickling process. In the early stage, the pH of egg white was increased rapidly, then declined, and the pH on a certain day of within 8-12 days of alkali-treatment reached the maximum; while the pH of egg yolk was kept increasing within 30 days, and it was lower than the egg white's during the same period [15,26]. The sharp increase of pH and prolonged pickling time contributed to the rapid formation of LAL in Chinese preserved egg white and yolk during the pickling period; however, the slow increase of LAL content in Chinese preserved egg white and yolk during the aging period mainly depended on the aging time.

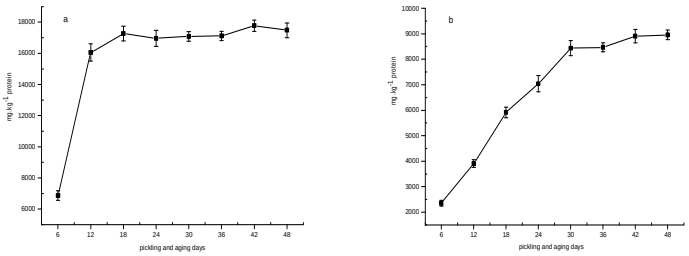

Fig.4 Changes of LAL in duck egg white (a) and yolk (b) of during alkali-pickling and storing processing

CONCLUSION

A simple, rapid, scientific and specific GC analysis method for LAL in Chinese preserved egg was developed. The results suggested that the amount of LAL in egg white was significantly elevated during the first 12 days of alkaline-pickling treatment, slowly increased in the end of pickling period and the aging process; but in yolk it was significantly raised during all the alkaline-pickling treatment, and it progressively elevated as well during the aging process. The study produced reliable data to support food safety risk assessment. As it investigated in this paper, the level of LAL both in egg white and yolk is quite high, and in order to produce Chinese preserved egg, 20-35 days of alkaline pickling is needed. Therefore, other ways instead of shortening processing time for minimizing the level of LAL in Chinese preserved egg remain to be further studied.

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