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A Rapid Method for Direct Determination of Levulinic Acid in Soy Sauce

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ABSTRACT

A simple and rapid method was developed to determine the levulinic acid level in soy sauce using megapore polar column (CP-Wax, 30 m × 0.53 mm) with splitless direct injection gas chromatography. Direct quantitative analysis of levulinic acid in soy sauce was carried out without any sample pretreatment procedure. A water-soluble compound, 1,6-hexanediol, was used as the internal standard. The detection limit for levulinic acid was 10 μg/mL. Recoveries from soy sauce and pickled condiment liquid were performed by spiking 2.5, 5.0, or 10.0 mg to 1 mL of test samples and were found to be at the range of 98~103% with coefficients of variation less than 6.3%. Fourty-six food samples including animal and vegetable protein hydrolysate, pickle condiment liquid, and soy sauce were analyzed using the proposed method. Levulinic acid contents in pickled condiment liquid were found to be 2.1~4.7 mg/mL and in soy sauce were 7.8~24.5 mg/mL, which were higher than CNS regulated level (1.0 mg/mL), indicating commercial pickles and soy sauce might be adulterated with vegetable protein hydrolysate. These results were inconsistent with the labeling of "100% fermented soy sauce" on the packages.

Key words: **soy sauce, levulinic acid, direct injection, gas chromatography, quantitive analysis.**

INTRODUCTION

Traditional soy sauce is made from soybean or black bean and wheat as protein source and carbohydrate source, respectively, while maintaining high concentration of salt (16~18%). After a long period of microbial fermentation, protein and carbohydrate are transformed into a mixture of amino acids, sugars, organic acids, alcohols, and esters. The mixture possesses a characteristic flavor, which is hardly present with other condiments. The type of soy sauce made by a rapid fermentation or blending process is called chemical soy sauce or vegetable protein hydrolysate, which is in high demand today(1). This is because the fermentation process for making traditional soy

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sauce is time-consuming and high in cost and can hardly satisfy the market demands. Making chemical soy sauce from acid hydrolysis is cheap and fast. However, the flavor of chemical soy sauce is poor. To compromise the dilemma, a product mixing chemical and traditional soy sauce is thus produced and commercialized[1-2].

Commercial soy sauce labeled as "100% fermented soy sauce" mostly are blended soy sauce. Their unit prices and qualities vary. Therefore, it is necessary to develop a rapid and precise analytical method to routinely analyze the amount of chemical soy sauce (vegetable protein hydrolysate) in commercial products.

Vegetable protein hydrolysate is produced by hydrolyzing the vegetable protein with hydrogen chloride at a high temperature of 100-130°C. During hydrolysis, levulinic acid is generated from a reaction between sugar and acid. In addition to protein and sugars, vegetable protein contains some lipids, which may be catalyzed by hydrogen chloride to form 3-chloro-1,2-propanediol (MCPD) and 1,3-dichloro-2-propanol (DCP)[3]. These two by-products have been shown to have toxic effects on human body. The mutagenicity, acute and chronic toxicity as well as infertility induced by MCPD and DCP have been also reported[5-10]. The current food technology is still unable to remove these two by-products from vegetable protein hydrolysate, which is why it is banned in western countries[3]. Detection of DCP and MCPD is a way to determine if soy sauce is adulterated with vegetable protein hydrolysate liquid. However, this detection method, which involved steam distillation and gas chromatography(GC) analysis[12-14] for determination of DCP and MCPD in soy sauce, is time-consuming and complex.

In addition to DCP and MCPD, the contents of levulinic acid can also be used as an index of soy sauce adulteration[15-21]. Levulinic acid contents in purely fermented soy sauce and chemical soy sauce are below 0.1% and higher than 2%, respectively. Vegetable protein hydrolysate liquid can be easily determined by checking levulinic acid concentration levels[22]. Current methods used to analyze levulinic acid include spectrophotometry[16], gas chromatography[15,18-19], paper chromatography[17], and high performance liquid chromatography[15]. However, sample pretreatment for the above methods is time-consuming and complex. Beside, interference from other organic acid could lead to inaccuracy in quantification. It is therefore necessary to set up a simple, rapid, and accurate method to determine levulinic acid in food. In this study, a simple and rapid gas chromatographic analytical technique with sample direct injection was expected to used to analyze levulinic acid in food.

MATERIALS AND METHODS

I. *Materials*

Domestic or imported soy sauce products including grade A soy sauce (12 samples), low-salt soy sauce (6 samples), and fermented fish sauce (5 samples) were purchased from local supermarkets of Tainan and Pintung. One purely fermented soy sauce sample was obtained from a soy sauce company in Pintung. Pickle condiment liquids (12 samples) were purchased from supermarkets in Tainan. Chemical soy sauce samples (10 brands) including hydrolyzed vegetable protein (HVP) and hydrolyzed animal protein (HAP) were supplied by Soy Sauce Association. Standards of levulinic acid, 1,5-pentanediol, 3-methyl-1,5-pentanediol and 1,6-hexanediol were obtained from Tokyo Chemical Inc.

II. *Methods*

(I) *Preparation of Vegetable Protein Hydrolysate (Chemical Soy Sauce)*[19]

One hundred g of defatted soybean and 3 volumes of 3 N HCl were placed in a 500-mL serum bottle, which was then autoclaved at 121°C for 2 hours. The hydrolysate was then neutralized by adding 3 N NaOH. The vegetable protein hydrolysate was thus prepared.

(II) *Preparation of Standard and Internal*

Standard Solutions

Levulinic acid (0.5 g) and 1,6-hexanediol (0.5 g) were separately weighed into a 100-mL volumetric bottle. Distilled water was then added to volume.

(III) *Determination of Relative Response Factor (RRF) of Levulinic Acid to 1,6-Hexanediol*

Standard solutions of levulinic acid and 1,6-hexanediol (internal standard) were prepared with levulinic acid/1,6-hexanediol ratios of 5/2, 2/1, 1/1, and 1/2. The RRF of levulinic acid to 1,6-hexanediol was calculated as follows based on the peak areas on GC analysis.

$RRF = (A_S/W_S)/(A_{IS}/W_{IS})$ .....(1),

where A_S is the peak area of levulinic acid; A_{IS} is the peak area of 1,6-hexanediol; W_S is the amount (mg) of levulinic acid; W_{IS} is the amount (mg) of 1,6-hexanediol.

(IV) *Quantification of Levulinic Acid*

One mL of soy sauce sample was placed in a 7-mL vial with cap. One mL of 0.5% (w/v) internal standard (1,6-hexanediol) was then added. The mixture was homogenized and 0.1 μL of which was then injected into GC device. The amount of levulinic acid was calculated according to a formula as follows:

Levulinic acid (mg/mL) = (A_S/A_{IS}) X (W_{IS}/RRF) X 1/V .....(2),

where V is the volume (mL) of test sample.

(V) *Test for Limit of Detection (LOD)*

Levulinic acid standard solution (0.5 mg/mL) was diluted to a series of concentrations of 100, 50, 25, 10, and 5 μg/mL. One mL of each dilution was spiked with 0.1 mL of 0.5% (w/v) internal standard (1,6-hexanediol). The mixture was then vortexed prior to GC analysis. LOD test for each concentration was carried out in triplicate.

(VI) *Fortification Recovery Test*

Levulinic acid standard with concentrations of 10, 5, and 2.5 mg/mL was individually spiked into a 7-mL vial, which contained 1 mL of soy sauce or pickled condiment liquid. One mL of 0.5% (w/v) internal standard (1,6-hexanediol) was added into the vial, which was then vortexed and 0.1 μL of mixture was then injected into the GC device. The test for each fortification was performed in triplicate and blank sample without standard fortification was also carried out. The recovery test was thus conducted.

(VII) *GC Conditions*

A Hitachi G-3000 GC equipped with FID detector was used in this study. The flow rates for hydrogen and air were 30 and 300 mL/min, respectively. The flow rate of carrier gas (He) was at 4 mL/min. A CP-Wax column (30 m X 0.53 mm i.d., 1 μm, Chrompack, Netherlands) was used. Oven temperature was kept at 150°C for 2 min and then programmed to rise to 220°C at 8°C/min followed by another rise to 250°C at a rate of 50°C/min. The injection volume was 0.1 μL and the injection mode was splitless direct injection.

RESULTS AND DISCUSSION

I. *Study on Chromatographic Conditions*

Multiple extraction by organic phase has been widely used recently for the quantification of levulinic acid in soy sauce[19-20]. The extract was then concentrated and analyzed by GC analysis. Lee *et al*[19] proposed a method to achieve 90% recovery of analyte. In their method, 1 mL of soy sauce was acidified to pH 2.0 and extracted with 10-fold diethyl ether for 3 times. The combined extracts were then concentrated to 1 mL and analyzed by GC with split injection mode. It took 30~40 min to finish one sample run. In our previous study[20], an analytical method, which was able to complete one sample run within about 20 min and give a high recovery ranged from 92 to 98% of levulinic acid, was developed. In our previous method, 5 mL of soy sauce was adjusted to pH 2.0 followed by saturation with NaCl and extraction with 6-fold ethyl acetate. The organic phase was collected, spiked with heptanoic acid (internal standard), and

rotary-evaporated under vacuum to remove ethyl acetate prior to GC analysis with splitless direct injection mode. The above two methods were convenient and fast. However, They require large volume solvent extraction to yield a recovery higher than 90% due to a highly hydrophilic property of levulinic acid.

GC method, with its high resolution and sensitivity, is one of the important analytical techniques used in modern world. It has been found that by inserting glass wool (or quartz wool) to glass liner in injection port or place a guard column (about 1~2 m) prior to analytical column, one is able to effectively prevent non-volatile compounds from contaminating the analytical column, as well as reduce the interference from contaminants, and decrease the tailing effect on peaks so as to increase the resolution on GC chromatogram[22-24]. Our previous study also showed that commercial megapore GC columns were highly water vapor-durable[24]. The resolution and retention time were comparable to those of a new column even after the aqueous solution was repeatedly injected into GC. Cleaning the glass liner was not necessary until more than 100 sample injections and the cleaning procedure was easy to follow. Just take out the glass liner, immerse in HCl solution for 10 min, remove the glass wool from glass liner, rinse with water and dry, replace with new glass wool (or quartz wool), and then re-install the glass liner back to GC injection port.

Our study was dependent on the GC performance as mentioned above. Soy sauce sample spiked with certain amount of internal standard was directly injected to GC without sample pretreatment. With this method, it's essential to select an adequate analytical GC column and GC programming to enable the levulinic acid in soy sauce or related products to be rapidly and precisely analyzed.

With respect to column selection, our study showed that a CP-Wax polar column, was suitable to analyze levulinic acid, a polar compound. A splitless direct injection mode was used. GC oven programming was based on the report of Lee *et al.* [19]. The initial oven temperature was 90°C kept for 2 min. It was then heated up to 200°C at 7.5°C/min (oven program 1). The detector and injector temperatures were set at 280°C and 250°C, respectively. Retention time of levulinic acid using oven program 1 was at 16.89 min as shown in Figure 1A. It took about 20 min to complete one sample run. The retention time of levulinic acid was further reduced to 9.47 min (Figure 1B) by changing the program to the oven program 2 as follows: initial temperature at 150°C kept for 2 min and then programmed to 220°C at a rate of 8°C/min. Analysis time was reduced to 10 min per sample run.

Regarding the internal standard selection, 3 aqueous standard solutions, 1,5-pentanediol, 3-methyl-1,5-pentanediol, and 1,6-hexanediol, were individually spiked to soy sauce or pickled condiment liquid sample and analyzed using the above GC condition. Results showed that the retention times of the above three compounds were 6.43, 7.11, and 7.55 min, respectively. 1,6-Hexanediol was found to be the best choice because the peak of which was completely resolved from other peaks in blank samples of soy sauce and pickled condiment liquid. Resolution was still satisfactory even when the initial oven temperature was increased from 90 to 150°C. 1,6-Hexanediol was therefore selected as internal standard in this study.

The above GC analytical conditions were thus used to analyze commercial products including grade A soy sauce, low-salt soy sauce, pickled condiment liquid, and HVP. GC chromatograms for the above products are demonstrated in Figure 2 A~D. The present method allowed a sample to be analyzed in 10 min.

II. *Relative Response Factor (RRF) of Levulinic Acid to Internal Standard*

1,6-Hexanediol, a water-soluble compound, was used as internal standard to quantify the levulinic acid in soy sauce. Determination of RRF of levulinic acid to internal standard was necessary for a quantification of the analyte in test samples. Levulinic acid content was calculated according to Formula(2). Figure 3 shows the calibration

curve of levulinic acid plotted by the peak areas of levulinic acid to 1,6-hexanediol versus concentrations. A satisfactory linearity with regression coefficient (R^2) higher than 0.99 was observed. RRF was determined to be 0.32.

III. *Detection Limit of Levulinic Acid*

Without sample pretreatment, test samples such as soy sauce were directly injected into GC-FID with splitless direct injection mode. Signal was set to FID = 1 and attenuation = 3. (The baseline of GC chromatogram was seriously shifted if the attenuation was set at lower than 2). The detection limit was determined to be 10 μg/mL as shown in Table 2.

IV. *Fortification Recovery Test*

Fortification recoveries of levulinic acid from soy sauce and pickled condiment liquid were shown in Table 3. Results showed that recoveries were within the ranges of 98~103% and 99~103% from soy sauce and pickled condiment liquid, respectively, at a spiking level of 10, 5.0, or 2.5 mg/mL. Coefficients of variation (CV%) were less than 6.3%. The method developed here was simple and easy to follow. Analyzing takes only 10 min for one sample run. So far, this is the fastest method among those found in the literature for the levulinic acid analysis. It took 30 min for a method involving diethyl ether as extraction solvent coupled with GC analysis developed by Lee *et al.*[19]. A previous study from our laboratory using ethyl acetate as extraction solvent accompanied with GC analysis took 15~20 min to accomplish one sample run[21]. It also took 30 min to complete one sample run using a HPLC reported by Yeh and Hsu[15]. The present method was therefore recommended for a routine analysis of

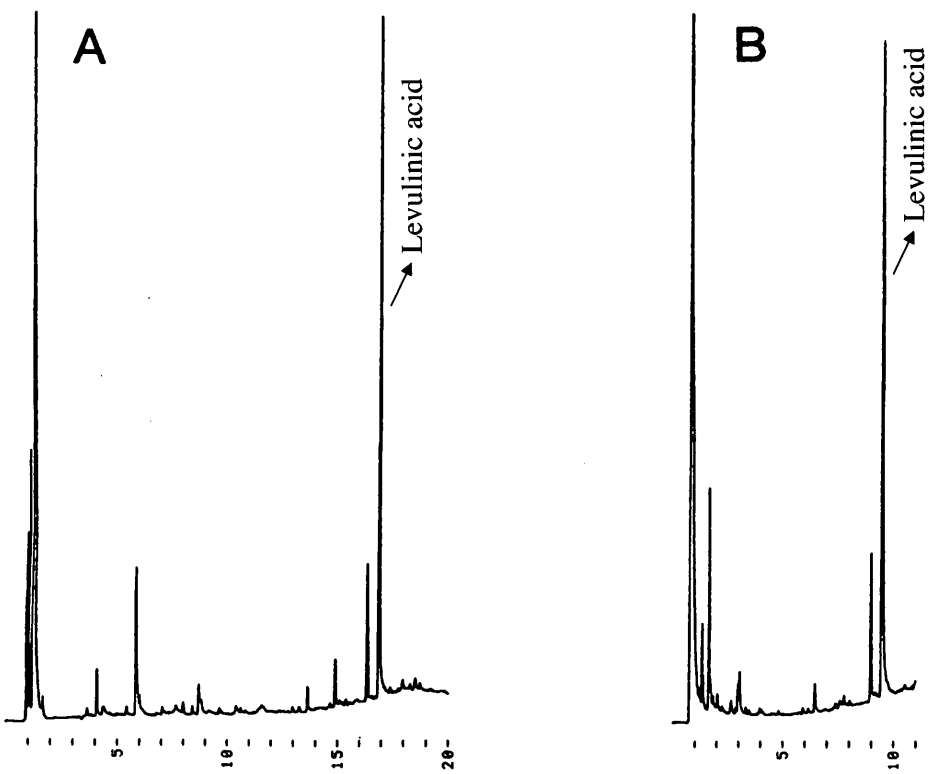

Figure 1. Gas chromatograms of levulinic acid of soy sauce by splitless direct injection method.
(A) Oven program 1 :
90°C (2 min) @ 7.5°C/min R220°C (2 min).
(B) Oven Program 2 :
150°C (2 min) @ 8°C/min R220°C @50°C/minR250°C.

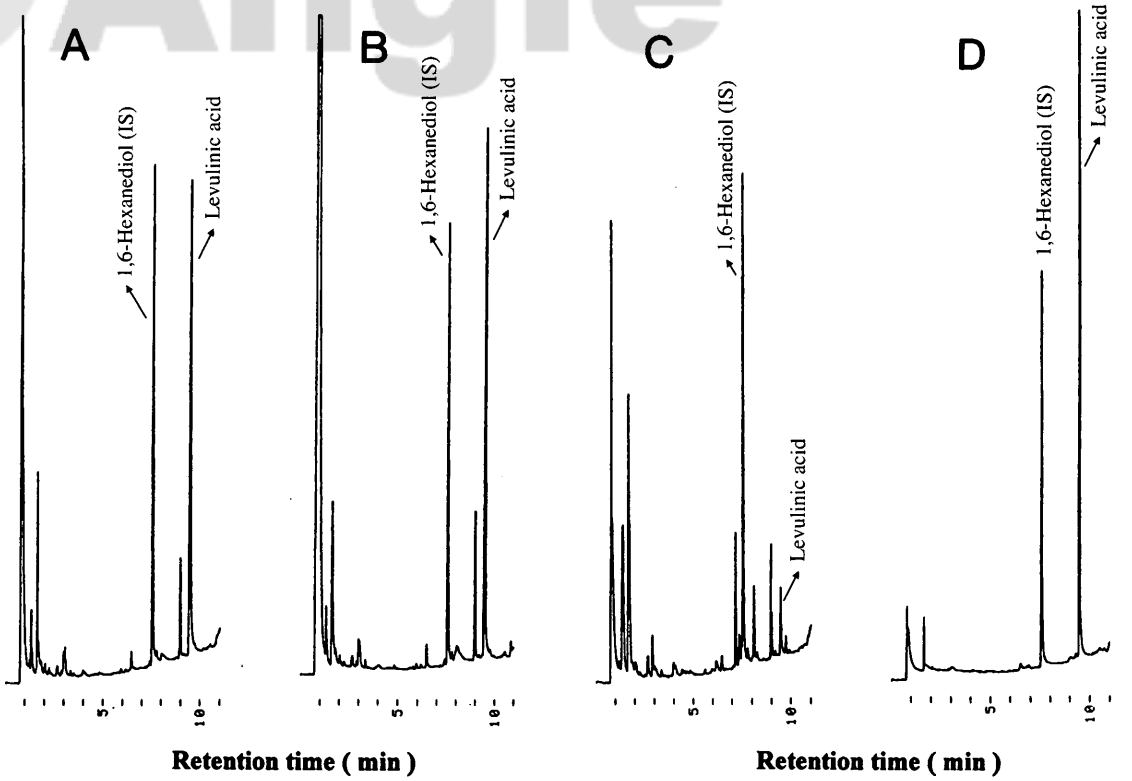

Figure 2. Gas chromatograms of levulinic acid of (A) grade A soy sauce, (B) low salt soy sauce, (C) pickled condiment liquid, (D) vegetable protein hydrolysates, (HVP) by splitless direct injection method.

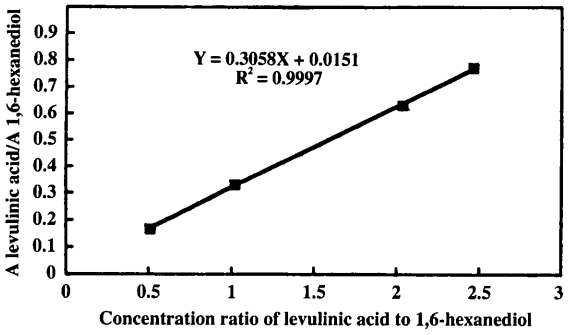

Figure 3. The calibration curve of levulinic acid to 1,6-hexanediol.

Table 1. Gas chromatographic retention time of levulinic acid and the internal standards

Compound	Retention time (min)[a]
1,5-Pentanediol	6.43
3-Methyl-1,5-pentanediol	7.11
1,6-Hexanediol	7.55
Levulinic acid	9.47

[a] CP-wax column (0.53 mm × 30 m) was used. Oven condition=150°C(2min) @ 8°C/min → 220°C @50°C/min → 250°C.

levulinic acid.

V. *Levulinic Acid Contents in Animal and Vegetable Protein Hydrolysates*

Totally 11 test samples, seven HVP, three HAP, and one defatted soybean hydrolysate samples[19] were separately injected to GC for levulinic acid analysis. Results were listed in Table 4. Levulinic acid contents in HVP ranged over 25~31 mg/mL were significantly higher than those in HAP ranged over 5~8 mg/mL. This could be due to the fact that sugar content in animal protein was less than that in vegetable protein resulting in a lower level of levulinic acid generated during acid hydrolysis process[21].

VI. *Levulinic Acid Contents in Commercial Soy Sauce and Pickled Condiment Liquid*

Results for the analysis of 12 commercial pickled condiment liquid samples were shown in Table 5. Levulinic acid contents were within the range of 2.1~4.7 mg/mL indicating those commercial products could be made of HVP.

Table 6 lists levulinic acid contents in 12 grade A soy sauce (including those labeled "100% pure fermented" or "high purity fermented" soy sauce), 1 soy sauce blank, 6 low-salt (LS) soy sauce, and 5 fish sauce samples, in total 24 test samples. Results showed that levulinic acid contents in grade A soy sauce were within the range of 7.8~24.5 mg/mL. Levulinic acid contents in labeled "100% pure fermented" or "high pure fermented" soy sauce samples were within the range of 7.8~19.4 mg/mL, which were less than the levels in other grade A samples but still much higher than the concentration level (0.87 mg/mL) in blank sample, a genuine purely fermented soy sauce. Levulinic acid contents in low-salt soy sauce and fish sauce products were within the range of 16.5~21.0 mg/mL and 1.1~8.4 mg/mL, respectively (Table 6). Much lower levels of levulinic acid were detected in fish sauce due to lower levulinic acid content in HAP, which was a material used for making fish sauce. However, the detected levels in fish sauce were still higher than a concentration of 0.87 mg/mL detected in blank sample.

According to the above results, levulinic acid contents in 12 grade A soy sauce samples were far higher than the CNS standard (1 mg/mL) by 8~24 folds, while levulinic acid contents in 6 low-salt soy sauce samples were even higher than the CNS standard (0.1 mg/mL) by 165~210 times. These results indicated both domestic and imported "purely fermented" soy sauces, which levulinic acid contents should be less than 0.1%[21], were adulterated with chemical soy sauce by 1/2 to 2/3. Soy sauce labeled "100% pure fermented" or "high purity fermented" soy sauces, whose levulinic acid contents should be less than 0.1%[21], were also adulterated with chemical soy sauce by 1/2 or less.

Levulinic acid levels in 12 grade A soy sauce samples ranged over 0.8~2.5% analyzed using the

Table 2. The detection level of levulinic acid using gas chromatography with FID detector

Levulinic acid content (μg/mL)[a]	Recovery (%)[b]	RSD (%)[c]
100	102.4	4.6
50	98.5	3.7
25	105.4	6.5
10	111.3	12.4
5	146.7	21.3

[a] FID range=1, Attenuation=3.

[b] Average of three analyses.

[c] Coefficient of variation (cv%).

Table 3. Recoveries of the levulinic acid in spiked commercial soy sauce and pickled condiment liquid by spitless direct injection method

Sample	Blank [a] (mg) (A)	Amount added (mg)(B)	Amount found [b] (mg) (C)	Recovery (%)[c]	CV (%)[d]
Soy sauce	13.94	10.00	24.77	103.47	5.44
	13.94	5.00	18.54	97.89	3.91
	13.94	2.50	16.81	102.25	5.82
Pickled	2.46	10.00	12.59	101.04	3.62
	2.46	5.00	7.37	98.79	5.7
	2.46	2.50	5.09	102.62	6.3

[a] Levulinic acid in 1 mL soy sauce and pickled condiment liquid.

[b] Average of three analyses.

[c] Recovery(%)=(C − A)/ B×100%.

[d] Coefficient of variation (cv%).

Table 4. Levulinic acid content of animal and vegetable protein hydrolysates (HAP & HVP)

Protein hydrolysate[a]	Content (mg/mL) [b]
HVP-1	28.28
HVP-2	26.75
HVP-3	31.13
HVP-4	27.89
HVP-5	31.19
HVP-6	25.47
HVP-7	26.17
HVP-8[c]	27.84
HAP-1	7.94
HAP-2	6.46
HAP-3	5.27

[a] From different soy sauce factories.
[b] Average of two analyses.
[c] Defatted soybean was hydrolyzed with 3N HCl at 121°C for 2 h.

Table 5. Levulinic acid content of some commercial pickled condiment liquid (PJ)

Sample	Content (mg/mL) [a]
PJ-1	2.56
PJ-2	2.22
PJ-3	4.66
PJ-4	2.45
PJ-5	2.12
PJ-6	3.74
PJ-7	3.61
PJ-8	3.89
PJ-9	3.49
PJ-10	3.98
PJ-11	2.61
PJ-12	3.55

[a] Average of two analyses.

present method showed no significant difference from the HPLC and GC methods reported by Yeh and Hsu[15]. They found levulinic acid contents in commercial soy sauce products to be over the range of 0.6~2.6%. The method developed in this study, however, was simple and quick. It allowed direct injection of a liquid sample into a GC device without pretreatment and the time for one

Table 6. Levulinic acid content of commercial soy sauce

Sample [a]	Content (mg/mL)[b]
Blank	0.87
S-1*	21.29
S-2 *	20.79
S-3 *	24.53
S-4 *	16.81
S-5 **	13.91
S-6 **	17.43
S-7 *	19.37
S-8 **	12.96
S-9 **	7.75
S-10 **	11.39
S-11 **	14.24
S-12 *	19.67
LS-1	20.67
LS-2	21.02
LS-3	19.36
LS-4***	20.94
LS-5 ***	16.51
LS-6 ***	19.78
FS-1	2.84
FS-2	8.43
FS-3	1.14
FS-4 ***	3.58
FS-5 ***	2.69

[a] Blank=pure fermented soy sauce; *A grade soy sauce; ** " labeled 100% fermented soy sauce"; *** imported products;
LS = low salt soy sauce.; FS = fish sauce.
[b] Average of two analyses.

sample was only 10 min. The present method can be used for a routine analysis. Yeh and Hsu's method, which required sample pretreatment, was complicated and time-consuming. It took more than 1 hour to accomplish one sample run. Therefore, using their method for a routine analysis was not suggested.

CONCLUSIONS

In this study, 0.1 μL of liquid samples such as

soy sauce spiked with a water-soluble internal standard (1,6-hexanediol) was directly injected into GC for levulinic acid analysis. GC oven temperature was kept at 150°C for 2 min, and then programmed to rise to 220°C at 8°C/min followed by another increase to 250°C at 50°C/min. This method was easy to follow and able to rapidly and precisely analyze levulinic acid in food in 10 min. Using this method, 46 commercial samples including HVP, HAP, grade A soy sauce, low-salt soy sauce, and pickled condiment liquid products were analyzed. Levulinic acid contents in grade A soy sauces were found to be 8~24 mg/mL, which were beyond the CNS national standard. Based on levulinic acid contents in test samples, commercial soy sauces, in general, were found to be a mixture of traditionally fermented soy sauce and chemical soy sauce.

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醬油中果糖酸之快速定量分析

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摘 要

本研究建立了直接注入氣相層析分析醬油中果糖酸之快速、簡便測定方法。採用直接注入(splitless direct injection)方式，以極性之CP-wax管柱(30 m × 0.53 mm)分析定量醬油中之果糖酸，選擇水溶性之1,6-己二醇 (1,6-hexanediol) 爲內標準，最低檢出量爲10 μg/mL左右。添加果糖酸2.5、5.0及10.0 mg/mL於1mL檢體中，直接注入GC分析，其回收率爲98～103%，變異係數在6.3%以下，顯示直接注入法之精確性高，且檢體不需經前處理即可直接分析，簡單且快速。以本方法分析不同廠牌之動、植物蛋白質酸水解液11件、醬菜醬瓜類之調味液12件及醬油23件，共46件之果糖酸含量。醬菜醬瓜調味液之果糖酸含量2.1～4.7 mg/mL及醬油之果糖酸含量7.8～24.5 mg/mL。此結果顯示國內、外醬油及醬菜醬瓜均有使用植物蛋白質酸水解液之情形。且醬油中果糖酸之含量已遠超過CNS中國國家標準(1 mg/mL)。由市售純釀醬油中之果糖酸含量顯示，與標示『100%純釀造』不符。

關鍵詞：醬油，果糖酸，直接注入法，氣相層析，定量分析。