Available online at www.sciencedirect.com
ScienceDirect
Food Chemistry 105 (2007) 1242–1247
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods

# Determination of levulinic acid in soy sauce by liquid chromatography with mass spectrometric detection

Atsushi Sano *, Tsuneo Satoh, Tetsuya Oguma, Akinori Nakatoh, Jun-ichi Satoh, Toshifumi Ohgawara

*Research and Development Division, Kikkoman Co., 399 Noda, Noda, Chiba 278-0037, Japan*

Received 15 December 2006; received in revised form 1 March 2007; accepted 3 March 2007

**Abstract**

A novel method using liquid chromatography coupled with mass spectrometry (LC–MS) was developed for the determination of levulinic acid (LV) in soy sauces to identify the addition of acid hydrolyzed vegetable protein (acid-HVP). One hundred percentage naturally brewed soy sauce (NBS) and enzymatic hydrolyzed vegetable protein (enzymatic HVP) did not contain LV (<0.01 mg/mL). There was apparent detection of LV in acid-HVP and blended soy sauce with acid-HVP (2.98–21.66 mg/mL). Gas chromatography (GC) and high performance liquid chromatography (HPLC) at 430 nm methods were also investigated. The results by GC gave similar data to those by LC–MS. In enzymatic HVP, LV was detected by HPLC at 430 nm, but it was confirmed that the detected component was not in fact LV by mass spectrometric identification of the isolated peak compound. We found that LV is under the detection limit in enzymatic HVP. This study showed that LV is below the detection limit in NBS and enzymatic HVP and is apparently detected in acid-HVP and blended soy sauces with acid-HVP. These results indicate that LV is a practical index of blend with acid-HVP and also corresponds with the JAS (Japanese Agricultural Standard) or CNS (Chinese National Standard) criteria in which the presence of LV in soy sauces indicates adulteration with acid-HVP. The LC–MS method is analytically optimal for the precise determination of LV, because peak misidentification may be practically eliminated.


*Keywords:* Levulinic acid; Determination; Soy sauce; Hydrolyzed protein; LC–MS

## 1. Introduction

Soy sauce is traditionally prepared by months of enzymatic brewing of a mixture of cooked soybeans and roasted wheat delation, thereby attaining a superiority in odor and taste. The production process can be speeded up by using acid hydrolysis of the defatted soybeans or wheat gluten rather than brewing. Some soy sauces are economically prepared as a blend of traditionally brewed soy sauce and acid-hydrolyzed vegetable delation protein. During the production of acid-hydrolyzed vegetable or soya protein (acid-HVP), however, certain carcinogenic compounds are generated such as 3-chloro-1,2-propanediol (Brereton et al., 2001) or 1,3-dichloropropanol (Hasnip et al., 2005). Therefore, such compounds are an important issue in the accurate discrimination among 100% naturally brewed soy sauce (NBS), acid-HVP, and blend of NBS and acid-HVP.

Qualitative tests that use levulinic acid (LV) to discriminate NBS and acid-HVP include a vanillin-$H_2SO_4$ method (JAS, 1978) and thin layer chromatography (Chen, Lin, & Sun, 1971). LV in acid-HVP originates from the acidic action on carbohydrates in soybeans (Manley & Fagerson, 1970). LV is very stable and has no poisonous or mutagenic properties. Under the conditions usually employed, i.e., 6 N HCl, 110 °C, and 24 h, the content of LV reaches 1000 mg/g total nitrogen in acid-HVP (Chei & Dun, 1976), depending upon the level of carbohydrates in

* Corresponding author. Tel.: +81 4 7123 5529; fax: +81 4 7123 5550.
*E-mail address:* asano@mail.kikkoman.co.jp (A. Sano).

0308-8146/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2007.03.004

materials such as soybeans. LV is the main source of unpleasant acid flavor in acid-HVP (Harada, 1971; Tanaka, Soejima, Aoyama, & Yagi, 1968). Carbonyl philic resins (Tanaka et al., 1968) or microorganisms (Harada, 1971) have been reported to remove a portion of LV from acid-HVP, but their use is economically impractical. NBS does not contain LV and has no additives. Therefore, LV is a proper index for determination of the acid-HVP content in blended soy sauce (BS).

Quantitative instrumental analysis to determine LV in soy sauce include the carboxylic acid analyzer method (Kanbe, Ozawa, Noda, & Sasaki, 1975), high performance liquid chromatography (Yeh-Chen & Hsu, 1985; Yoshida, Ogura, & Yoshino, 1981), and gas chromatography (Hirose & Fukuzaki, 1975). The specific LV level in NBS is reported to be undetectable with the carboxylic acid analyzer method (Kanbe et al., 1975), high performance liquid chromatography (HPLC) (Yeh-Chen & Hsu, 1985; Yoshida et al., 1981), or gas chromatography (GC) (Hirose & Fukuzaki, 1975, 1976; Yamashita, Udaka, & Tamura, 1972), so that the presence of LV has been considered suitable as an index for discrimination between NBS and acid-HVP. However, different results were reported in recent years that LV is detected in NBS with a range of 2.0–19.4 mg/mL by means of GC (Wang, Lin, Lee, & Choong, 1999; Crews, Le Brun, Hough, Harvey, & Brereton, 2000). The disadvantages of GC include the result that some compounds coeluate with the intended component after many injections and analyses, and might not be identified correctly just by the retention times. It is consequently necessary to develop a new analytical method for LV with higher accuracy and precision.

There is no regulatory limit for chloropropanols in soy sauce in some countries, but the Committee on Carcinogenicity of Chemicals in Food, Consumer Products and the Environment (COC) has recommended that exposure should be reduced to as low a level technologically feasible. Producers of acid-HVP have modified the hydrolysis conditions and post-hydrolysis treatments in recent years to achieve substantial reductions in the levels of chloropropanols present in acid-HVP. However, adulteration takes place in NBS with acid-HVP dilute carcinogenic compounds. Therefore, an accurate index is needed to evaluate the additive rate of acid-HVP.

In this paper, we describe the development of a highly precise and sensitive quantitative method to determine LV in soy sauces by liquid chromatography coupled with mass spectrometry (LC–MS). We also investigated the usefulness of two reported methods in HPLC (Yeh-Chen & Hsu, 1985) and GC (Wang et al., 1999; Crews et al., 2000) and compared LC–MS so as to verify the accuracy of determining LV in soy sauce. Besides NBS and acid-HVP, the recently developed enzymatic hydrolyzed wheat gluten soy sauce is also measured for LV levels. This allows an estimation of the acid-HVP amounts blended with NBS in commercially available soy sauces. Moreover, the determined LV amounts identify the test samples as to whether they are NBS or acid-HVP or a blended acid-HVP with NBS.

## 2. Materials and methods

### 2.1. Soy sauce samples

NBS (22 samples) were purchased from seven soy sauce manufacturers in Japan, three in Taiwan, and one in UK. Acid-HVP or blended soy sauces (14 samples) were from four manufacturers in Japan, three in Taiwan, and one in the USA. Enzymatic hydrolyzed soy sauces (six samples) were provided by two manufacturers in Japan and Singapore.

### 2.2. Reagents

LV was purchased from the Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Acetonitrile and distilled water for LC–MS grade were from Kanto Chemical Co., Inc. (Tokyo, Japan) and formic acid of HPLC grade from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Doubly distilled water for the dilution of samples was purified using a Milli-Q system (Millipore, Bedford, MA).

### 2.3. LC–MS

The HPLC system was an Alliance 2690 equipped with an autosampler, degassor, sample cooler, and heater column purchased from the Waters Co. (Milford, MA). The mass spectrometer system was a ZQ tandem quadrupole (Quattro micro API mass analyzer) also purchased from the Waters Co. (Milford, MA). Data were collected by MassLynx ver.4.0 software in a personal computer. The HPLC column was an Atlantis $dC_{18}$ 2.1 mm i.d. × 150 mm 5 μm (Waters, Milford, MA) and was set to 28 °C during all running procedures, and the injection volume was 5 μl. Two mobile phases were used to generate a linear gradient with a 0.2 mL/min flow-rate. Mobile phase A was water with 0.01% (v/v) formic acid, and mobile phase B was acetonitrile with 0.01% (v/v) formic acid. The acid presence was essential to chromatographic efficiency of LV, specifically the elimination of peak tailing. The linear gradient was from 0% to 10% B for the first 12 min and from 10% to 50% B for the next 3 min; mobile phase B was kept at 50% for 3 min and then reduced to 0% for 1 min; finally the column was equilibrated in 0% B for 21 min. For the first 5 min and from 12 min onward, the eluent was directed to waste to avoid the contamination of the mass spectrometer with salts or washout components. LV was detected with an electrospray ionization probe (ESI) in negative mode. The source temperature was 150 °C, the flows for desolving and cone voltage gas were 600 L/h and 50 L/h, respectively, the capillary voltage was set to 3.2 kV, and cone voltage was adjusted to 18 V. Selective ion monitoring (SIM) was acquired by a previous infusion of a standard in full scan mode at a concentration

of 0.05 mg/mL. Twenty-fold dilution of soy sauce samples was typically applied for the LC–MS analysis after membrane filtration using a cellulose acetate filter with a 0.45 μm pore size (Toyo Roshi Kaisha, Ltd., Tokyo, Japan). When the detected LV is beyond 0.1 mg/mL, which is the upper limit of the LV standard curve, further dilution of samples is needed, i.e. an extra 20-fold dilution.

### 2.4. GC

LV levels in soy sauces by GC were determined using the method of Wang et al. with a slight modification. An Agilent 5890 GC equipped with a flame ionization detector was used in this study. To reduce the injection nozzle blockage caused by deposition of ingredients, each soy sauce sample was diluted 10 times with distilled water for GC measurement. Other conditions were as described in the method of Wang et al.

### 2.5. HPLC at 430 nm

LV levels in soy sauces by HPLC at 430 nm were determined using the post-column method of Yen-Chen and Hsu with a slight modification. LC detection with a variable UV detector was performed using a pump CCPM-II (Tosoh Co., Tokyo, Japan), a variable UV detector UV-8020 (Tosoh Co., Tokyo, Japan), an autosampler AS-8020 (Tosoh Co., Tokyo, Japan), and a column oven AO50 (Showa Denko K.K., Tokyo, Japan). Data were collected by Tosoh multi station LC-8020 software in a personal computer. The cation exchange LC column was a 10–20 μm sulfonate type porous styrene–divinyl-benzene copolymer (Shodex RSpak KC-811, 8.0 mm i.d. × 300 mm) and the pre column was 20–30 μm, with the same packing material described above (Shodex Rspak KC-LG, 8.0 mm i.d. × 15 mm). The mobile phase used 3.5 mM $HClO_4$. The indicator reagent for the post-column method was a 10-fold diluted solution of ST3-R reagent (Showa Denko K.K., Tokyo, Japan). The mobile phase and indicator reagent were filtered (0.45 μm) before use. The flow rate was 0.8 mL/min in both the eluting solution and indicator reagent. The column temperature was maintained at 50 °C. Fivefold dilution of soy sauce samples were applied for analysis, after the membrane filtration and in the same manner described in the LC–MS preparation.

## 3. Results and discussion

### 3.1. Recovery test

The recovery of the LV added to NBS was measured by the three methods. Twenty-fold dilution recovery was obtained with the LC–MS and HPLC at 430 nm methods, but interferences were infrequently found when the GC method was used. To reduce peak overlap with HPLC at 430 nm, more than five times dilution with distilled water was determined to be practical. In the GC procedure, consecutive injections without sample dilution reported by Wang et al. caused deposition of ingredients at the sample inlet to lower the precision of the analysis. It was consequently necessary to perform 10-fold dilution with distilled water and blank injection at every sample injection.

### 3.2. Detection limit by LC–MS

For solution standards, dilutions were made in distillated water with 0.05% formic acid (v/v) to give the following concentrations: 0.100, 0.050, 0.020, 0.010, 0.005, 0.002, and 0.001 mg/mL. The standard curve was constructed by plotting the peak areas of the standard analytes versus the theoretical concentrations. The precision and accuracy of the LC–MS method were assessed within the entire concentration range. The limit of detection was 0.01 mg/mL in undiluted soy sauces as defined by the lowest sample concentration that could be quantified with good precision (relative standard deviation <20%).

### 3.3. Determination of LV in NBS

Table 1 shows the results of LV analysis in LC–MS, GC, and HPLC at 430 nm. LV was not detected in any of the tested NBS samples. This result indicates that LV is not present or is under the detection limit (i.e. <0.01 mg/mL) in NBS. It is supported by the LV being shown to be absent by spectrophotometric measurement (JAS, 1978), and to be under 1.0 mg/mL (CNS, 1983). In the past reports, there was no LV detected in NBS (Hirose & Fukuzaki, 1976; Kanbe et al., 1975; Ushijima, Hamada, & Kanbe, 1982;

Table 1
Result of LV levels in 100% naturally brewed soy sauces by three different methods, LC–MS, GC, and HPLC at 430 nm

| Sample no. | Producer country | LC–MS[a] (mg/mL) | GC[a] (mg/mL) | HPLC at 430 nm[a] (mg/mL) |
|---|---|---|---|---|
| 1 | Japan | n.d. | n.d.[b] | n.d. |
| 2 | Japan | n.d. | n.d. | n.d. |
| 3 | Japan | n.d. | n.d. | n.d. |
| 4 | Japan | n.d. | n.d. | n.d. |
| 5 | Japan | n.d. | n.d. | n.d. |
| 6 | Japan | n.d. | n.d. | n.d. |
| 7 | Japan | n.d. | n.d. | n.d. |
| 8 | Japan | n.d. | n.d. | n.d. |
| 9 | Japan | n.d. | n.d. | n.d. |
| 10 | Japan | n.d. | n.d. | n.d. |
| 11 | Japan | n.d. | n.d. | n.d. |
| 12 | Japan | n.d. | n.d. | n.d. |
| 13 | Japan | n.d. | n.d. | n.d. |
| 14 | Japan | n.d. | n.d. | n.d. |
| 15 | Japan | n.d. | n.d. | n.d. |
| 16 | Taiwan | n.d. | n.d. | n.d. |
| 17 | Taiwan | n.d. | n.d. | n.d. |
| 18 | Taiwan | n.d. | n.d. | n.d. |
| 19 | Taiwan | n.d. | n.d. | n.d. |
| 20 | Taiwan | n.d. | n.d. | n.d. |
| 21 | Taiwan | n.d. | n.d. | n.d. |
| 22 | UK | n.d. | n.d. | n.d. |

[a] Each point is the mean of three measurements.
[b] n.d.: not detected.

Yamada, Kumai, & Uchimi, 1974; Yamashita et al., 1972), or a small amount of LV was detected in NBS (Yeh-Chen & Hsu, 1985). The detected small amount of LV could come from contamination by residual acid-HVP from the manufacturing facility, such as a storage tank or a lead pipe (Yeh-Chen & Hsu, 1985). LV was not detected (<0.01 mg/mL) in any of the tested NBS in the present study, and this may be attributable to recent improvements in the quality management of the manufacturing process.

We followed the original analytical conditions of GC in which LV was detected in a range of 2.0–19.4 mg/mL in NBS (Wang et al., 1999; Crews et al., 2000), but the results were under the detection limit of the analysis method (<0.05 mg/mL) in all of the tested NBS samples (Table 1, Fig. 1a). In the GC chromatogram of NBS spiked with LV at 0.25 mg/mL, LV is recognized as a clear peak close to glycerol (Fig. 1b). It is reported that NBS made from defatted soybeans, wheat and salt solution contains glycerol in a range of 1.03–1.74% by GC analysis (Sakurai & Okuhara, 1977). A measurable glycerol should appear close to the LV peak in NBS spiked with LV as analyzed by the reported GC method (Wang et al., 1999). The GC chromatogram for the NBS sample shown in the previous report (Wang et al., 1999) might be due to an insufficient separation between the LV peak and the measurable glycerol peak.

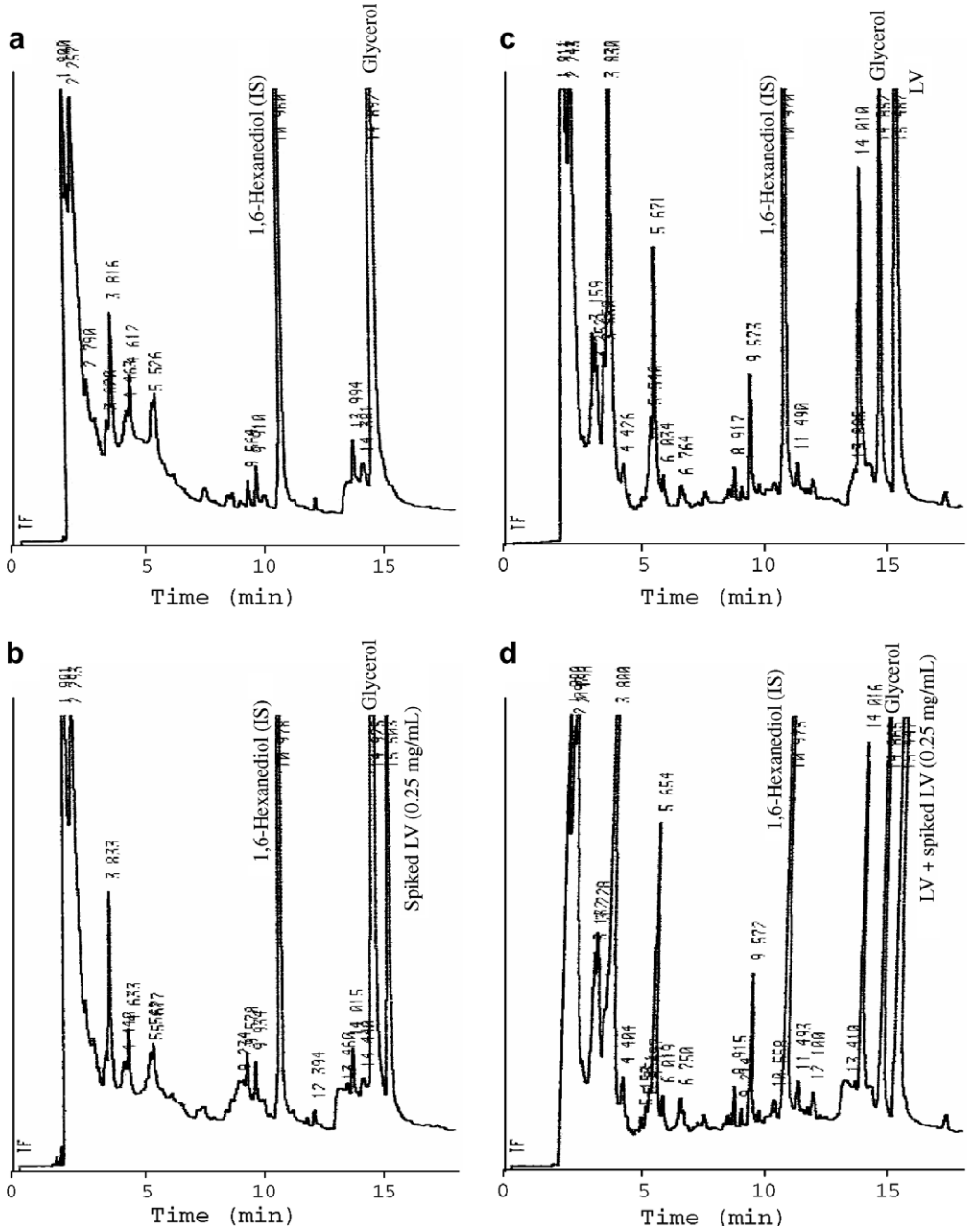


Fig. 1. Gas chromatograms of LV and glycerol of (a) NBS, (b) NBS spiked with 0.25 mg/mL of LV, (c) acid-HVP, and (d) acid-HVP spiked with 0.25 mg/mL of LV.

Table 2
Result of LV levels in acid-HVP or blended with acid-HVP in the three different methods, LC–MS, GC, and HPLC at 430 nm

| Sample no. | Producer country | LC–MS[a] (mg/mL) | GC[a] (mg/mL) | HPLC at 430 nm[a] (mg/mL) |
|---|---|---|---|---|
| 1 | Japan | 17.91 | 12.77 | 20.41 |
| 2 | Japan | 21.66 | 15.12 | 24.79 |
| 3 | Japan | 18.89 | 15.09 | 20.11 |
| 4 | Japan | 4.06 | 2.51 | 3.29 |
| 5 | Japan | 2.98 | 3.44 | 3.83 |
| 6 | Japan | 7.95 | 7.02 | 7.28 |
| 7 | Taiwan | 5.58 | 5.01 | 5.67 |
| 8 | Taiwan | 4.40 | 4.29 | 5.35 |
| 9 | Taiwan | 5.52 | 8.56 | 6.74 |
| 10 | Taiwan | 6.72 | 8.40 | 7.56 |
| 11 | Taiwan | 5.24 | 5.23 | 12.51 |
| 12 | Taiwan | 7.42 | 5.59 | 8.83 |
| 13 | USA | 10.85 | 9.06 | 10.03 |
| 14 | USA | 14.09 | 12.75 | 11.24 |

[a] Each point is the mean of three measurements.

Table 3
Result of LV levels in enzymatic hydrolyzed vegetable protein in the three different methods, LC–MS, GC, and HPLC at 430 nm

| Sample no. | Producer country | LC–MS[a] (mg/mL) | GC[a] (mg/mL) | HPLC at 430 nm[a] (mg/mL) |
|---|---|---|---|---|
| 1 | Japan | n.d.[b] | n.d. | 0.18 |
| 2 | Japan | n.d. | n.d. | 0.17 |
| 3 | Japan | n.d. | n.d. | 2.01 |
| 4 | Singapore | n.d. | n.d. | 3.29 |
| 5 | Singapore | n.d. | n.d. | 1.36 |
| 6 | Singapore | n.d. | n.d. | 2.75 |

[a] Each point is the mean of three measurements.
[b] n.d.: not detected.

### 3.4. Determination of LV in acid-HVP and estimation of acid-HVP in BS

LV was significantly detected in all 14 acid-HVP samples by the three analytical techniques (Table 2). This result is in agreement with the criteria of JAS and CNS that LV should be contained in acid-HVP or a blended soy sauce with acid-HVP. In this study, some samples contained LV at levels below 1.0 mg/mL, which is lower than the concentration in acid-HVP as noted in CNS. It is suggested that these samples were blended acid-HVP with other soy sauces such as NBS or enzymatic HVP. LV levels could be attenuated in blended items according to the reduction of the acid-HVP concentration. To estimate the concentrations of acid-HVP in BS, we prepared artificial blended soy sauces with an NBS (Sample no. 1 in Table 1) and an acid-HVP (Sample no. 1 in Table 2) in five different blend ratios (100:0, 75:25, 50:50, 25:75, 0:100, v/v), and determined the concentrations of LV. The detected LV in the laboratory-prepared BS is linear with respect to the additive rate of the acid-HVP with the NBS ($R^2 = 0.9990$). This linearity indicates an estimation of the additional acid-HVP in BS. The determination of LV could be an indicator of adulteration with acid-HVP because the sensitivity of LV in BS is adequate even to a level of 1% additional acid-HVP. Consequently, the detection of LV might suggest indirectly the presence of carcinogenic compounds such chloropropanols. The correlative value of LV and chloropropanols will be determined in future work.

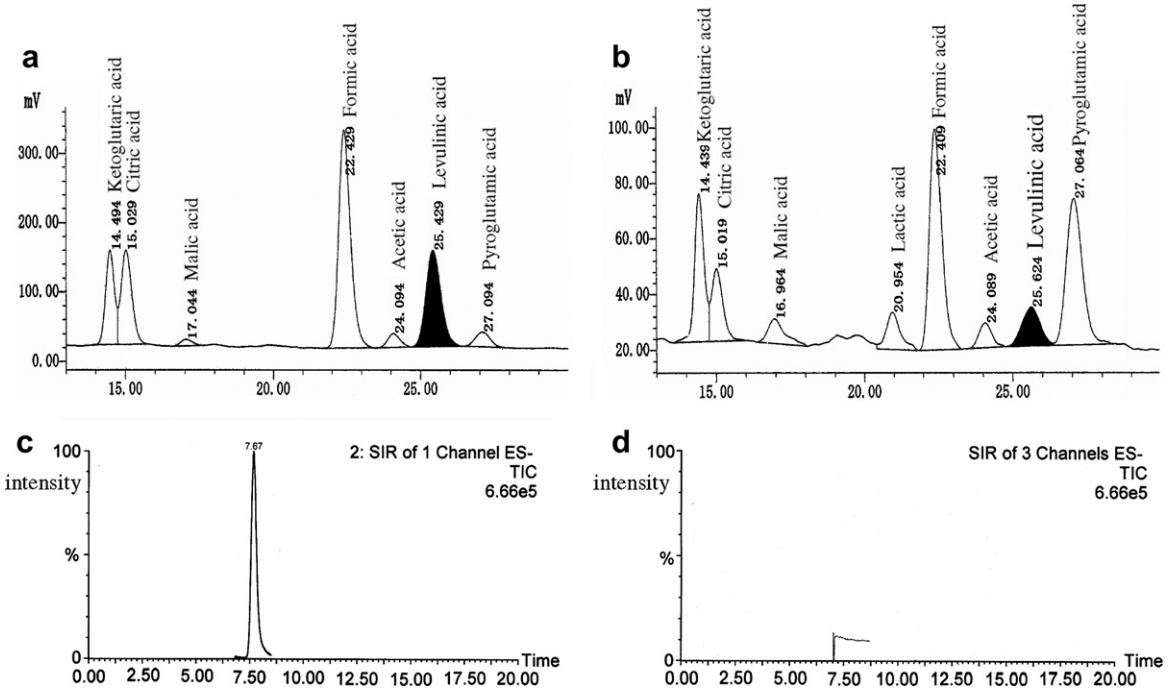


Fig. 2. (a) HPLC chromatogram at 430 nm of acid-HVP (Sample no. 13 in Table 2), (b) LC–MS chromatogram in selective LV ion monitoring mode of the isolated peak as LV in acid-HVP (Sample no. 13 in Table 2), (c) HPLC chromatogram at 430 nm of enzymatic HVP (Sample no. 4 in Table 3), (d) LC–MS chromatogram in selective LV ion monitoring mode of the isolated peak as LV in enzymatic HVP (Sample no. 4 in Table 3).

### 3.5. Determination of LV in enzymatic HVP

Table 3 shows the LV levels in enzymatic HVP samples determined by LC–MS, GC, and HPLC at 430 nm methods. The measurement results by the LC–MS and GC methods were under the detection limit of LV in enzymatic HVP, while different results by HPLC at 430 nm were obtained in a range of 0.17–3.29 mg/mL LV. LV production is less likely during the production process of enzymatic HVP because the acid hydrolysis process with strong acidity and heat is not employed. The LV detected by HPLC at 430 nm may be an interference that happened to coincide with the retention time of LV. To clarify the uncertainty of the detected LV peak by HPLC at 430 nm, the peak was isolated by preparative liquid chromatography and analyzed by LC–MS in SIM mode of LV (Fig. 2). The peak in the acid-HVP (Sample no. 13 in Table 2) was identified as LV by the retention time and the molecular mass by LC–MS (Fig. 2a and b). However, the peak in the enzymatic HVP (Sample no. 4 in Table 3) was revealed not to have $m/z$ 115, which is the negative ion of LV by LC–MS (Fig. 2c and d). This result indicates that the peak detected as LV by HPLC at 430 nm in enzymatic HVP was not LV but another compound with a similar retention time. The disadvantage of HPLC at 430 nm includes compounds that coeluate with LV during HPLC and might not be identified correctly when using a nonspecific detector such as the UV–VIS-detector. Meanwhile, the advantage of LC–MS includes peak identification by two-step analysis of the retention time and the mass spectrum. These analytical features suggest that the LC–MS method is more accurate and precise than HPLC at 430 nm for the determination of LV in complicated materials such soy sauces or HVP. This study revealed that LV is under the detection limit in enzymatic HVP.

## 4. Conclusion

A novel LC–MS method was developed to determine LV in NBS, acid-HVP, enzymatic HVP and BS with good accuracy and precision. This analysis showed that there was no LV in NBS or enzymatic HVP and there was apparent detection of LV in acid-HVP and blended soy sauces with acid-HVP. These results indicates that LV is a practical index of blending with acid-HVP and also corresponds with the JAS and/or CNS criteria in which the LV existence in soy sauces indicates adulteration with acid-HVP. Meanwhile, only HPLC at 430 nm gave LV detection in enzymatic HVP, which finding was, however, confirmed not to be LV by mass spectrometric identification of the isolated peak compound. It is necessary to take special care in the determination of LV by means of HPLC with a UV–VIS-detector or the GC method based only on the retention time in a complex materials such as soy sauce. The LC–MS method is analytically optimal for the precise determination of LV.

## References


Brereton, P., Kelly, J., Crews, C., Honour, S., Wood, R., & Davies, A. (2001). Determination of 3-chloro-1,2-propanediol in foods and food ingredients by gas chromatography with mass spectrometric detection: collaborative study. *Journal of AOAC International, 84*(2), 455–465.

Chei, W. J., & Dun, S. C. (1976). *Practical technology of soy sauce fermentation*. Taipei, Taiwan: Universal Publishing.

Chen, C. M., Lin, D. K., & Sun, C. F. (1971). *Study on discrimination between fermented soy sauce and synthetic soy sauce (acid hydrolysate of soybean), paper partition chromatographic method*. Research Paper No. 21, Taiwan, ROC: Food Industry R & D Institute.

Chinese National Standard (CNS), (1983). Test methods for soy sauce-levulinic acid analysis (paper chromatography). 955, N6008.

Crews, C., Le Brun, G., Hough, P., Harvey, D., & Brereton, P. (2000). Chlorinated propanols and levulinic acid in soy sauces. *Czech Journal of Food Science, 18*, 276–277.

Harada, M. (1971). Metabolism of levulinic acid by microorganisms Part IV. *Journal of Agricultural Chemistry Society of Japan, 45*(2), 89–95 (in Japanese).

Hasnip, S., Crews, C., Potter, N., Brereton, P., Diserens, H., & Oberson, J. M. (2005). Determination of 1,3-dichloropropanol in soy sauce and related products by headspace gas chromatography with mass spectrometric detection: interlaboratory study. *Journal of AOAC International, 88*(5), 1404–1412.

Hirose, Y., & Fukuzaki, K. (1975). Development of a new method of methylation and isolation, and gas chromatographic determination of organic acids in soy sauce (Part 1). *Journal of Japanese Soy Sauce Research Institute, 1*(4), 201–207 (in Japanese).

Hirose, Y., & Fukuzaki, K. (1976). Development of a new method of methylation and isolation, and gas chromatographic determination of organic acids in soy sauce (Part 2). *Journal of Japanese Soy Sauce Research Institute, 2*(1), 22–26 (in Japanese).

Kanbe, C., Ozawa, Y., Noda, F., & Sasaki, T. (1975). Determination of organic acids in soy sauce by a new carboxylic acid analyzer. *Journal of Japanese Soy Sauce Research Institute, 1*(3), 142–148 (in Japanese).

Japanese Agricultural Standards (JAS) Association, (1978). *Index of inspection on food sanitation II category of food*, pp. 67–68.

Manley, C. H., & Fagerson, I. S. (1970). Major volatile neutral and acid compounds of hydrolyzed soy protein. *Journal of Agricultural and Food Chemistry, 18*(3), 340–347.

Sakurai, H., & Okuhara, A. (1977). Rapid determination of glycerin in soy sauce by gaschromatography. *Journal of Japanese Soy Sauce Research Institute, 3*(6), 290–294 (in Japanese).

Tanaka, T., Soejima, M., Aoyama, T., & Yagi, N. (1968). Adsorption and elution of carbonyl compounds on C.P. resin. *Journal of Agricultural Chemistry Society of Japan, 42*(1), 49–54 (in Japanese).

Ushijima, S., Hamada, T., & Kanbe, C. (1982). A new liquid chromatographic determination of organic acids in soy sauce. *Journal of Japanese Soy Sauce Research Institute, 8*(2), 58–63 (in Japanese).

Yamada, K., Kumai, K., & Uchimi, N. (1974). Judgment of soy sauce quality by gas chromatography Part 2. *Seasoning Science, 21*(10), 27–30 (in Japanese).

Yamashita, I., Udaka, K., & Tamura, T. (1972). Studies on the analytical method of organic acids in foods Part II. *Journal of the Japanese Society for Food Science and Technology, 19*(5), 194–199 (in Japanese).

Yeh-Chen, S. L., & Hsu, C. T. (1985). Liquid chromatographic determination of protein acid hydrolysate content in blended soy sauce. *Journal of Association of Official Analytical Chemists, 68*(4), 618–621.

Yoshida, S., Ogura, M., & Yoshino, H. (1981). Determination of organic acids in soy sauce by high pressure liquid chromatography (Part 3). *Journal of Japanese Soy Sauce Research Institute, 7*(5), 225–229 (in Japanese).

Wang, M. L., Lin, H. J., Lee, M. H., & Choong, Y. M. (1999). A rapid method for direct determination of levulinic acid in soy sauce. *Journal of Food and Drug Analysis, 7*(2), 143–152.