# Negative and Positive Ion Mode LC/MS/MS for Simple, Sensitive Analysis of Sorbic Acid

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Received July 17, 2009; accepted October 9, 2009; published online October 14, 2009

**Sorbic acid (SA: $CH_3$–CH=CH–CH=CH–COOH) is one of the widely used food preservatives, although there have been some reports of its toxic activity, for example, on DNA and skin cells. In order to examine the effects of SA on mammalian tissues, we have developed a highly sensitive analytical method using LC/MS/MS with positive and negative ion mode electrospray ionization (ESI). In a previous study, we found that a nonacidic eluent offers better ionization efficiency than acids or their ammoniun salts. However, optimal results could not be obtained because the anion form of SA is poorly retained on a conventional reversed phase column. To resolve this problem, we chose a new type of column and used high-resolution mass spectrometry and positive ion mode analysis. There have only been a few reports using these methods in the positive mode, for example derivatized SA, because acid compounds such as SA are usually used in the negative ion mode. However, a new type of low-carbon-content and polar-endcapped C18 phase column was developed for better separation of SA from the matrix. High-resolution selected reaction monitoring (SRM) gave the best signal to noise ratio in normal-resolution SRM. In the positive ion mode, the $CH_3OH$–0.05% HCOOH/0.1% $CH_3COOH$ eluent system yielded the best ionization efficiency. We propose a highly sensitive and simple analysis using a two-ion-mode ESI SRM method. Such systems should allow quantification of the amount of SA in or around the cells, without the need for pretreatment such as solid phase extraction.**

**Key words** sorbic acid; LC/MS/MS; electrospray ionization; selected reaction monitoring

Sorbic acid (SA) and its salts are commonly used as food additives because of their antibacterial and growth inhibitory activities against yeast and fungi. They are also used in cosmetics, pharmaceuticals and tobacco products.[1)] Their usage for food preservation is usually considered to be safe for human consumption. However, some studies have shown that SA and its salts exhibit a weak genotoxic potential,[2—4)] including causing damage to DNA[5)] and having an alkylating activity on nucleophilic 4-(*p*-nitrobenzyl) pyridine.[6)] Also, Soschin and Leyden reported that SA induced erythema and edema in human skin,[7)] although the mechanism was not clarified.

There is a need to understand the actions of SA and its salts in terms of possible cytostatic or cytotoxic effects in mammalian tissues and cells. Some work has been done to examine the effects of SA on cultured mammalian cells.[8,9)]

Mass spectrometry, which offers high sensitivity and selectivity, should be ideally suited to the detection of SA. However, there are few reports on the use of LC/MS for SA analysis. Negri *et al.* reported that SA in urine could be measured by selected ion monitoring (SIM) of *m*/*z* 111.13 with electrospray ionization (ESI)-LC/MS in the negative ion mode with the detection limit of 4 $\mu$mol/l,[10)] and Cartwright *et al.* reported that SA derivative at less than 4 fmol was detected by using ESI-LC/MS/MS selected reaction monitoring (SRM) in the positive ion mode.[11)] Generally, the SRM mode is more selective and sensitive than the SIM mode, and the technique for detecting the SA derivative requires extra time for analysis because of unwanted side products.

In a previous paper, we reported a simple and sensitive method for the determination of SA in or around cultured mammalian cells by using neutral solvents ($CH_3OH$/$CH_3CN$–$H_2O$) for HPLC (eluents) under negative ion mode ESI-LC/MS/MS.[12)] Acids and their ammonium acetate solvents are usually used as eluents. However, this sacrifices the ionization efficiency of SA under negative ion mode ESI for retention on a conventional reversed phase column. On the other hand, the anion form of SA has high ionization efficiency under the negative ion mode ESI but poorly retains SA on a conventional reversed phase column, thus not giving the best separation of SA from the cytosol fraction.

To solve the problem, we developed simpler, more sensitive and better separation techniques for the determination of SA in the cells by using LC/MS/MS in the positive and negative ion modes. With the negative ion mode, we used two experimental approaches. First, HPLC columns were examined for their ability to retain and separate the anion form of SA from a matrix such as cytosol. The columns used were endcapped reversed phase columns (ODS), carbon-column and temperature-responsive silica columns filled with cross-linked poly (*N*-isopropylacylamide) hydrogel-modified amino silica beads.[13—15)] Second, a high resolution mass spectrometer was used in an SRM mode for mass separation of SA from the matrix using the exact mass value. We also tried positive ion mode ESI-LC/MS/MS. To find the most sensitive eluent system under the positive ion mode, we compared the use of acid solvents with the use of ammonium acetate.

To evaluate the matrix effect for the analysis of SA in the positive ion mode, we measured the recovery of SA from the cytosol of mastocytoma P-815 cells, which are used as a proper model of growing mammalian cells because P-815 cells are favorable for examining growth and differentiation, and also for the evaluation of various compounds with re-

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spect to cytotoxicity, phototoxicity and immunotoxicity.[16—22]

## Experimental

**Materials** SA, guaranteed grade, and LC/MS-grade of $H_2O$, $CH_3OH$ and $CH_3CN$ were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Guaranteed grades of HCOOH, $CH_3COOH$, $HCOONH_4$ and $CH_3COONH_4$ were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Argon gas (99.99%) used as the collision gas of the SRM mode was obtained from Neriki Gas Co., Ltd. (Hyogo, Japan).

**Preparation of Cytosol** Cytosol from the mastocytoma cells, preloaded with 2.5 mmol/l SA for 0.5 h or without SA, was prepared as described in a previous paper.[12] These extractions were analyzed by LC-MS/MS.

**Columns and Conditions** ODS columns, carbon column and cation column were used for the analysis of SA under negative ion mode ESI. ODS columns (4.6 mm i.d.×150 mm); Handy ODS (particle size 5 $\mu$m) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), TSKgel ODS-100V (particle size 3 $\mu$m) and TSKgel ODS-100Z (particle size 5 $\mu$m) were from TOSOH Co. (Tokyo, Japan), Hypersil GOLD aQ (particle size 3 $\mu$m) and Thermo Hypersil-Keystone BetaMax Neutral (4.6 mm i.d.×150 mm and 2.1 mm i.d.×150 mm; particle size 5 $\mu$m) were from Thermo Fisher Scientific K. K. (Yokohama, Japan). Carbon column Hypercarb (4.6 mm i.d.×150 mm; particle size 5 $\mu$m) was obtained from Thermo Fisher Scientific K. K. (Yokohama, Japan). The temperature response cation silica column, which used cross linked poly (*N*-isopropylacylamide) hydrogel-modified amino silica beads, Aqua Way Cation (4.6 mm i.d.×150 mm; particle size 5 $\mu$m) was obtained from CellSeed Inc. (Tokyo, Japan). The retention time of SA under negative ion mode of these columns and these eluents are summarized in Table 1. A Thermo Hypersil-Keystone BetaMax Neutral (2.1 mm i.d.×150 mm; particle size 5 $\mu$m) was used for the separation column, under negative ion mode ESI high resolution SRM. A binary mobile phase consisting of $H_2O$ (solvent A) and $CH_3CN$ (solvent B) was used in the following program: 0 min, 10% B; 2.0 min, 70% B; 8.0 min, 70% B; 8.1 min, 10% B, 15 min, 10% B. The flow rate was 0.2 ml/min, and the injection volume was 5 $\mu$l. Under the positive ion mode ESI-SRM, a TSK gel ODS 100 V was used as a separation column. The eluents used and ionization efficiencies are summarized in Table 2.

**Triple-Quadrupole Mass Spectrometer Conditions** A Quattro premier triple-quadrupole LC-MS (Micromass, Manchester, U.K.), equipped with an ESI source was used for the negative ion and positive ion mode MS/MS analyses coupled to the Alliance HT Waters 2795 separation module (Waters Co., Milford, MA, U.S.A.). The instrumental parameters of the negative ion mode were used as described in our previous paper.[12] Under the positive ion mode, the parameters of the ionization efficiency were optimized by evaluating the sensitivity based on flow injection analysis. SA, 1 mmol/ml, was injected at 5 $\mu$l/min by syringe and connected with the line of the mobile phase of 0.05% HCOOH–$CH_3OH$ (40 : 60), flow rate 0.4 ml/min, *via* a T-joint. The parameters of the analyzer were optimized under the same conditions, temperature (source and desolvation) and the nitrogen gas flow rate (cone and desolvation), of the negative ion mode. The product ion spectrum was obtained by scanning Q3 over the mass range of *m/z* 40—120. The flow rate of the argon collision gas for fragmentation in the SRM mode was 0.3 ml/min ($3.37—3.39\times10^{-3}$ mbar) by which the collisional energy was optimized for the fragment ion of SA. The optimized value of the cone was set at 19 V and the collisional energy at 10 eV. During the Q3 scan, the Low Mass (LM) and High Mass (HM) resolution values for both Q1 and Q3 quadrupoles were 15; while during SRM analysis, they were 10.

The high resolution SRM analysis under negative ion mode ESI was done using a TSQ Quantum Ultra (Thermo Fisher Scientific K.K., Yokohama, Japan) equipped with Prominence (Shimadzu Corp., Kyoto, Japan) HPLC system. The resolutions of Q1 and Q3 were set at 0.1 and 0.7 for high resolution SRM, respectively. The optimized parameters were: spray voltage 2000 V; vaporizer temperature, 350 °C; ion transfer tube temperature, 330 °C; sheath gas, 30 arbitrary units; auxiliary gas, 20 arbitrary units; skimmer collision-induced dissociation, 5 eV; collision gas, Ar; collision gas pressure, 0.6 m Torr.

Table 1. Retention Time of SA Using Various Columns

| Column | Eluent | Carbon content (%) | Retention time (min) |
|---|---|---|---|
| Handy ODS | $H_2O$–$CH_3CN$ (60 : 40)[a] | 16 | 5.1 |
| TSKgel ODS-100V | $H_2O$–$CH_3CN$ (60 : 40)[a] | 15 | 6.2 |
| TSKgel ODS-100Z | $H_2O$–$CH_3CN$ (60 : 40)[a] | 20 | 2.8 |
| Hypersil GOLD aQ | $H_2O$–$CH_3CN$ (60 : 40)[a] | 12 | 7.0 |
| Hypersil-Keystone BetaMax Neutral | $H_2O$–$CH_3CN$ (60 : 40)[a] | 29 | 3.2 |
| Hypercarb | 0.02% HCOOH–$CH_3CN$ (10 : 90)[a] | — | 8.2 |
| | 5 mM $HCOONH_4$–$CH_3CN$ (10 : 90)[a] | | 8.6 |
| | $H_2O$–$CH_3CN$ (5 : 95)[a] | | N.D. |
| Aqua Way Cation | $H_2O$–$CH_3CN$ (10 : 90)[b] | — | 8.2 |

*a*) Flow rate: 0.4 ml/min, column temperature: 30 °C. *b*) Flow rate 1.0 ml/min, column temperature: 40 °C. N.D.: not detected.

Table 2. Effect of Eluent on Ionization Efficiency of Positive Ion Mode ESI

| Eluent | pmol | Peak area | Peak area ratio |
|---|---|---|---|
| 0.05% HCOOH/2 mM $HCOONH_4$–$CH_3CN$ | 2.5 | 112 | 1 |
| 0.05% HCOOH/2 mM $HCOONH_4$–$CH_3OH$ | 2.5 | 1297 | 11.6 |
| 0.05% HCOOH–$CH_3CN$ | 2.5 | 8211 | 73.3 |
| 0.05% HCOOH–$CH_3OH$ | 2.5 | 15842 | 141.4 |
| 0.1% $CH_3COOH$–$CH_3CN$ | 2.5 | 11308 | 101.0 |
| 0.1% $CH_3COOH$–$CH_3OH$ | 2.5 | 20515 | 183.2 |

The volumes of $CH_3CN$ and $CH_3OH$ were 50% and 60%, respectively. Flow rate: 0.4 ml/min.

## Results and Discussion

**Negative Ion Mode ESI Analysis** Under the negative ion mode, we examined two strategies to solve the problem of poorly retained SA on ODS columns. First, we compared the retention time of SA among the five ODS columns and other types of column under isocratic eluent conditions (Table 1). In the case of ODS columns, SA is more strongly retained on Handy ODS, TSKgel ODS-100V and Hypersil GOLD aQ columns (Fig. 2A), than TSKgel ODS 100Z and Hypersil-Keystone BetaMax Neutral columns. The carbon content levels of these columns (%) were 16%, 15%, 12%, 20% and 29%, respectively and their accessible silanol groups were endcapped with various reagents, *i.e.* TSKgel ODS-100V with difunctional dialkylsilane reagents and Hypersil GOLD aQ with polar functional group(s). Hypercarb, one of the carbon columns, did not retain SA with neutral eluent. The Aqua Way Cation column, which responds to temperature and other external stimuli, showed good separation of SA from the cytosol fraction (Fig. 2B), but the sensi-

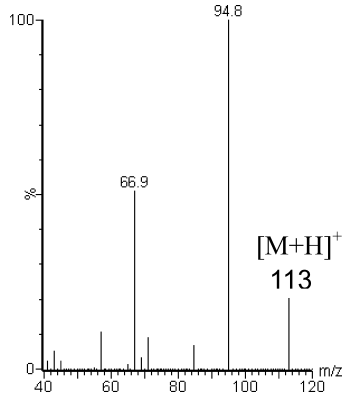


Fig. 1. Positive Ion Mode ESI Product Ion Spectra of $[M+H]^+$ *m/z* 113 with Optimized Collisional Energy

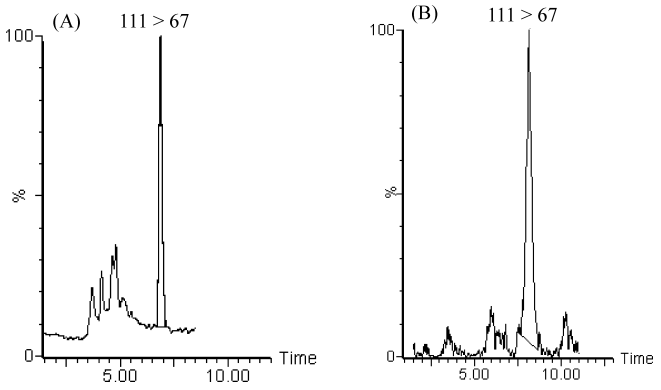


Fig. 2. Negative Ion Mode ESI SRM Chromatogram of Cytosol Fraction Spiked with SA

(A) An equal volume of SA 102.5 nmol/l was added to filtered cytosol, and 20 $\mu$l was injected to LC/MS . Eluent: $CH_3CN$–$H_2O$ (40 : 60), flow rate: 0.4 ml/min, column: Hypersil GOLD aQ. (B) An equal volume of SA 1.0 $\mu$mol/l was added to filtered cytosol, and 20 $\mu$l was injected to LC/MS. Eluent: $CH_3CN$–$H_2O$ (90 : 10), flow rate: 1 ml/min, column: Aqua Way Cation.

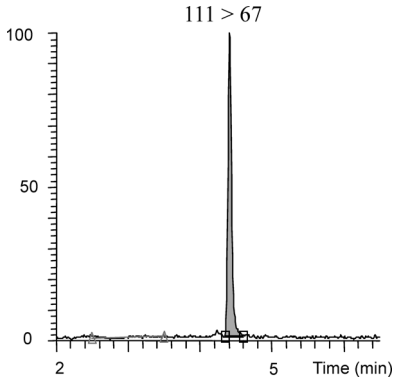


Fig. 3. Negative Ion Mode ESI High Resolution SRM Chromatogram of Cytosol Fraction Spiked with SA

An equal volume of SA 102.5 nmol/l was added to filtered cytosol, and 5 $\mu$l was injected to LC/MS (SA 256 fmol, S/N 81). Eluent: $CH_3CN$–$H_2O$ (10—70 : 90—30), flow rate: 0.2 ml/min, column: Thermo Hypersil-Keystone BetaMax Neutral.

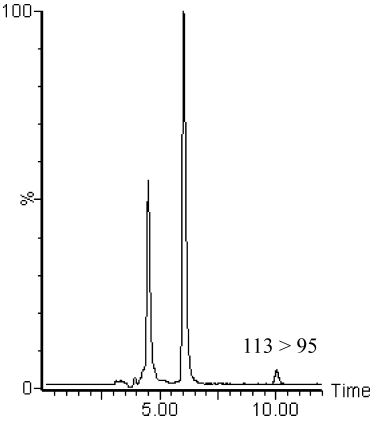


Fig. 4. Positive Ion Mode ESI SRM Chromatogram of Cytosol Fraction Spiked with SA

An equal volume of SA 102.5 nmol/l was added to filtered cytosol, and 5 $\mu$l was injected to LC/MS (SA 256 fmol, S/N 24). Eluent: 0.05%HCOOH–$CH_3OH$ (40 : 60), flow rate: 0.4 ml/min, column: TSKgel ODS-100V.

tivity was poor, owing to the character that SA was tightly connected with the cation function on silica beads. These results indicated that low carbon % and polar endcapped C18 phase columns would be useful as separation columns for SA under neutral conditions. These polar endcapped columns were suitable for polar compounds, providing evidence for the reasonableness of these results. The carbon column (Hypercarb) did not provide good results for the anion form of SA. The cation column (Aqua Way Cation) utilizes temperature-responsive polymer as the stationary phase. The retention mechanism of SA on this column was very different from the case of cytosol fraction. SA flows out faster with 10% $CH_3OH$ than 20% $CH_3OH$, but cytosol fraction came out faster with 10% $CH_3OH$ than 5% $CH_3OH$. Using a 10% $CH_3OH$–$H_2O$ eluent system, we achieved excellent separation between the two compounds, but poor recovery efficiency of SA. Second, for another approach to the analysis of the anion form of SA, we used a high resolution mass spectrometer. This mass spectrometer is capable of separating SA from cytosol matrices based on mass accuracy. Thus, the S/N of the SRM chromatogram was improved (Fig. 3), and a good quantitation limit was obtained. The linearity was good up to 5000 fmol ($r^2$=0.9991), and the detection limit was 5 fmol (S/N 3). The high resolution mass spectrometer was useful for identification as well as quantification of the analytes.

**Positive Ion Mode ESI Analysis** The negative ion mode ESI is usually used for SA analysis in trying to find the most sensitive eluent under the negative ion mode, we discovered that the neutral condition was suitable for highly sensitive detection of SA because it took the anion form at pH 7. However, the anion form of SA showed poor retention on ODS columns. This is the dilemma of negative ion mode analysis of SA. We therefore decided to try using a positive ion mode analysis for SA.

**Optimization of MS and MS/MS Conditions** MS conditions were optimized according to the previous paper. The capillary voltage was 3.5 kV and cone voltage was 19 V. The temperature of the source and desolvation were 120 °C and 350 °C, respectively. The gas flow rate (1/h) of the cone and desolvation were 100 and 1000, respectively. The product ion mass spectrum of the protonated molecular ion $[M+H]^+$ $m/z$ 113 is shown in Fig. 1; $m/z$ 95 appeared at the production mass spectrum when the collision energy was set at 10 eV. The mass transition pattern $[M+H]^+$ $m/z$ 113→95 was selected to monitor SA in the positive ion mode. To obtain the most sensitive ionization conditions for analysis of SA under the positive ion mode, we compared the peak area of $m/z$ 113→95 in SRM method using various eluents. For positive ion analysis, 0.05% HCOOH–$CH_3OH$/$CH_3CN$, 0.1% $CH_3COOH$–$CH_3OH$/$CH_3CN$, 0.05% HCOOH–2 mM $CHOONH_4$–$CH_3OH$/$CH_3CN$ was used as the eluent. The peak area of 2.5 pmol SA (500 nM of SA was injected at 5 $\mu$l) was compared for the different eluent systems, and the results are summarized in Table 2. The results indicated that $CH_3OH$ was better than $CH_3CN$ (1.9 times under 0.05% HCOOH, 1.8 times under 0.1% $CH_3COOH$) and 0.1% $CH_3COOH$ was better than 0.05% HCOOH (1.3 times under $CH_3OH$, 1.4 times under $CH_3CN$). The acetate buffer containing eluent did not yield good results (low peak area).

**Sensitivity and Matrix Effect** Using these optimized eluent conditions, 0.05% HCOOH and 0.1% $CH_3COOH$/$CH_3OH$, calibration was performed. The calibration graph for

SA was generated from the peak areas of the mass transition pattern, $[M+H]^+$ $m/z$ 113→95 in SRM method (SRM chromatogram is shown in Fig. 4). A calibration curve was constructed using the least-squares method of quantities *versus* peak area. The linearity was good up to 25 pmol ($r^2=0.9989$ for 0.05% HCOOH, $r^2=0.9996$ for 0.1% $CH_3COOH$), and the detection limit (S/N 3) was 35 fmol for 0.05% HCOOH, 25 fmol for 0.1% $CH_3COOH$. Next, the matrix effect was evaluated by comparing the peak areas of the cytosol blank spiked with SA to those prepared in the mobile phase at the corresponding concentration. A linear calibration curve was constructed using the same method mentioned above. Good linearity was obtained up to 1250 fmol ($r^2=0.9978$ for 0.05% HCOOH, $r^2=0.9991$ for 0.1% $CH_3COOH$) and the detection limit (S/N 3) was 35 fmol for 0.05% HCOOH, 25 fmol for 0.1% $CH_3COOH$. In a previous study, we detected SA at 160 fmol/$5\times10^6$ cells ($n=3$) content in the cytosol of P-815 cells treated with SA (2.5 mmol/l) for 0.5 h under the negative ion mode.[12] The positive ion mode ESI analysis was shown to be useful for quantification and separation of this volume of SA under the matrix, in mammalian cells.

## Conclusions

In this study, we established a highly sensitive analysis method for SA in the cytosol fraction. In the negative ion mode ESI SRM method, a low carbon content and polar end-capped C18 phase ODS columns showed better retention time for the ion form of SA than other ODS columns. A column with a novel separation mechanism (Aqua Way Cation column) facilitated analysis of the ion form of SA from the cytosol fraction, but its ability to maintain the ion form of SA was too high resulting in a low rate of collection of the ion form of SA. Therefore, this column could not be used for highly sensitive quantification of the ion form of SA, but could be used for analysis to determine the amount of SA as an additive. The SRM chromatogram obtained using a high resolution mass spectrometer led to a higher S/N than the usual SRM chromatogram for analysis of SA in the cytosol fraction. Under the positive ion mode ESI SRM method, using the 0.1% $CH_3COOH$ and 0.05% HCOOH–$CH_3OH$ system as an eluent of HPLC gave a higher peak area of SA than 0.1% $CH_3COOH$, 0.05% HCOOH–$CH_3CN$ and 0.05% HCOOH/2 mM $HCOONH_4$–$CH_3OH$/$CH_3CN$. Addition of the acid to the eluent system is effective for enhancement of $[M+H]^+$ under the positive ion mode, because $H^+$ adheres to the molecular form of SA in the acid eluent. This is the first demonstration of use of the positive ion mode ESI SRM method for highly sensitive analysis of the acidic compounds such as SA.

In positive ion mode, normal resolution SRM showed a better detection limit than in the negative ion mode, and it was better than separating SA using conventional ODS columns. Mass separation analysis of an analyte from a matrix using a high resolution mass spectrometer is effective for obtaining a clear S/N SRM chromatogram and a good detection limit.

**Acknowledgments** This study was supported in part by a Health Science Research Grant from the Ministry of Health, Labour and Welfare, Japan. We would like to thank Ms. M. Yamaguchi and Ms. K. Nakagawa (Thermo Fisher Scientific K. K.) for the high resolution analysis of SA using TSQ quantum Ultra, and Ms. N. Kato (Waters Co.) for her useful suggestions and technical support. We would also like to thank Dr. K. Sasaki (TOSOH Co.), Dr. O. Hiroaki (Thermo Fisher Scientific K. K.) and Dr. D. Suga (CellSeed Inc.) for their support of our study.

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