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Article

HUMAN NUTRIENT METHODS

Determination of Sorbic Acid in Cheese by High Performance Liquid Chromatography

Jackie E. Wood, Brendon D. Gill, Gaurav D. Gujaran, Harvey E. Indyk, and Peter M. White

Fonterra Co-operative Group Ltd, P.O. Box 7, Waitoa 3341, New Zealand

Corresponding author's e-mail: jackie.wood@fonterra.com

Abstract

Background: Sorbic acid (*E, E*-2, 4-hexadienoic acid) is added as a preservative to cheese because of its fungistatic and antimicrobial activity.
Objective: A facile method for the analysis of sorbic acid that is applicable to sliced processed cheese and grated cheese products.
Method: A cheese sample and dry-ice mixture was blended and sorbic acid was extracted with methanol and analyzed by HPLC-ultraviolet with external standardization. A large sample size was used to overcome sample inhomogeneity due to imprecise sorbic acid addition techniques during production and sorbic acid migration through the fat over time.
Results: The method was shown to be accurate for both processed cheese and grated Cheddar cheese, with acceptable spike recovery (93.7, 103.7%, respectively), and no bias ($\alpha = 0.05$) against an international reference method ($p = 0.59$, $p = 0.13$, respectively) was found. Acceptable precision was confirmed for both processed cheese slices and grated Cheddar cheese, with repeatability of 5.3% and 4.3% relative standard deviation, respectively, and intermediate precision Horwitz ratio values of 1.3 and 1.7 for processed cheese slices and grated Cheddar cheese, respectively. Method detection limit and ruggedness experiments further demonstrated the suitability of this method for routine compliance testing.
Conclusions: A method for high-throughput, routine testing of sorbic acid is described. The method was subjected to single-laboratory validation and was found to be accurate, precise, and fit-for-purpose.

Sorbic acid (*E, E*-2, 4-hexadienoic acid) can exist as four possible geometric isomers, which can interconvert under ultraviolet (UV) irradiation, however only *E, E*-2, 4 isomer occurs naturally (1).

Sorbic acid's antimicrobial activity was first discovered in 1939 (2) and, since the mid-1950s, sorbic acid has been used in a variety of foods as a preservative because of its anti-mold, anti-yeast, and antibacterial activities (2, 3), with the naturally occurring isomer exhibiting the highest antimicrobial activity (1). Sorbic acid, or its potassium, sodium, or calcium salts, is added to a wide range of foods including jams and jellies, margarine, butter, processed cheese and meats, bakery items (bread, cakes, pies), dried fruits and fruit juices, and wine (3–5).

Sorbic acid was initially used to protect block cheese by applying it to the wrapper. However, it was found that it migrated into the cheese, lessening its fungistatic activity at the surface of the cheese (6). In current manufacturing practice, sorbic acid or its salt either is melted into processed cheese slices when it is being processed, or is sprinkled on the surface of shredded cheese. Currently in New Zealand, sorbic acid is not used in the majority of block cheeses available in the supermarket.

New Zealand and Australian food regulations state that 3000 mg/kg of sorbic acid can be added into processed cheese or sprinkled on to the surface of shredded cheese, whereas the European regulatory level is lower at 1000 mg/kg (4, 7). The international limit for sorbic acid in processed cheese is 3000 mg/kg (8). As sorbic acid and sorbates are rapidly catabolized

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through β-oxidation, they have low toxicity to humans, with the only known adverse health effect being contact urticaria, through its use as a preservative in cosmetics (3, 9).

In the 1950s, Melnick and Luckmann (10) developed an analytical method for sorbic acid in cheese, utilizing spectrophotometry after steam distillation from the cheese sample. Spectrophotometric methods after steam distillation were still used until the 1970s, although the resulting extract was oxidized to cope with interfering compounds derived from the cheese (11). GC has also been used to analyze sorbic acid either using silylated derivatives (11, 12) or by directly injecting into the GC–mass spectrometry (MS) instrument (13). More recently, a number of LC–UV methods for sorbic acid have been developed, usually coupled with another preservative, benzoic acid, for a range of beverages and foodstuffs including cheese (14–24). Two LC–MS methods have been developed to quantitate sorbic acid in hard, ripened, pasta filata, and fresh cheeses (25, 26).

The analysis of sorbic acid in many cheeses is challenging because of their high fat content; the sorbic acid migrates into the fat phase and this introduces complications in obtaining a homogeneous representative test sample (6). Although some reported methods involve heating as part of the sample preparation (21), this can be problematic, because sorbic acid sublimes at or above 60°C (27).

Therefore, the purpose of this study was to develop and single-laboratory validate a simple, high-throughput method for the routine analysis of the sorbic acid content in cheese using HPLC with UV detection.

METHOD

Apparatus

(a) *Pipettes*.—Eppendorf Research plus, 20 200, and 1000 μL (Hauppauge, NY) or equivalent.
(b) *Centrifuge*.—Heraeus Multifuge X3 centrifuge (ThermoFisher, Waltham, MA) or equivalent.
(c) *Centrifuge tubes*.—Polypropylene, 50 mL (ThermoFisher, Waltham, MA) or equivalent.
(d) *Microcentrifuge tubes*.—Polypropylene, 2 mL (Eppendorf, Hamburg, Germany) or equivalent.
(e) *Vortex mixer*.—Genius 3 (IKA, Wilmington, NC) or equivalent.
(f) *Analytical balance*.—Mettler-Toledo (Columbus, OH) AE 260 analytical delta range (±0.1 mg) or equivalent, calibrated with National Institute of Standards and Technology (NIST; Gaithersburg, MD) traceable calibration weights.
(g) *HPLC column*.—Luna PFP 150 mm × 4.6 mm, 5 μm (Phenomenex, Torrance, CA) or equivalent.
(h) *HPLC system*.—Prominence HPLC system consisting of two LC-20AT pumps, an SIL-20AC autosampler, a CTO-20AC column oven, a CBM-20A control module, an SPD-M20A photodiode array detector, and a DGU-20A5R degasser unit. Lab Solutions software version 5.73 (Shimadzu, Kyoto, Japan) or equivalent.
(i) *Bottletop pump dispenser*.—25 mL.
(j) *Graduated cylinders*.—100 and 1000 mL.
(k) *Volumetric flasks*.—10, 50, and 100 mL.
(l) *HPLC injection vials*.—Amber, 1 mL with Teflon-coated caps.

Reagents

(a) *Acetic acid (CH_3COOH)*.—HPLC grade or equivalent.
(b) *Ammonium acetate (NH_4CH_3COO)*.—Reagent grade or equivalent.
(c) *Methanol (CH_3OH)*.—HPLC grade or equivalent.
(d) *Water (H_2O)*.—HPLC grade or equivalent.
(e) *Sorbic acid (E, E-2, 4-hexadienoic acid)*.—Purity ≥99.0% (Sigma-Aldrich, St. Louis, MO) or equivalent.
(f) *Dry ice (solid CO_2)*.

Solutions

(a) *Acetate buffer (1.5 mol/L, pH = 4.6)*.—Dissolve 15.0 g of ammonium acetate in ~800 mL of water, add 15 mL of acetic acid, and dilute to 1 L with water.
(b) *Buffer: methanol mix*.—Mix 2 parts of acetate buffer to 1 part of methanol.
(c) *Mobile phase A*.—Acetate buffer.
(d) *Mobile phase B*.—Methanol, 100% v/v.

Standards

A sorbic acid stock standard (5 mg/mL) was made by weighing 0.504 g of sorbic acid into a 100 mL volumetric flask and diluting to volume with methanol.

Samples

Two types of consumer cheese were investigated: (i) grated Cheddar-style cheese with sorbic acid applied to the surface; (ii) unwrapped sliced processed cheese with integrated sorbic acid.

Sample Preparation

Approximately 200 g of cheese with an equal or greater portion of dry ice was added to a blender and ground into small granules. After allowing any remaining CO_2 to sublimate, approximately 1 g of cheese powder was weighed accurately into a 50 mL disposable centrifuge tube. Using a pump dispenser, 25 mL of methanol was added and blended with an Ultra-Turrax for 60 s. The samples were then placed in a centrifuge at 1600 × *g* for 10 min. A 50 μL aliquot of the supernatant was transferred to a 2 mL microcentrifuge tube containing 950 μL of buffer: methanol mix, vortex mixed for 20 s, and then centrifuged at 10 000 × *g* for 5 min. The upper ~0.8 mL portion of this extraction was transferred to an HPLC vial ready for analysis.

LC–UV Analysis

(a) *HPLC system*.—*See Apparatus* (**h**).
(b) *HPLC column*.—Luna PFP 150 mm × 4.6 mm, 5 μm (Phenomenex).
(c) *Column temperature*.—25°C.
(d) *Injection volume*.—10 μL.
(e) *Binary gradient*.—Settings in Table 1.
(f) *UV detection*.—254 nm.

Table 1. Chromatographic gradient conditions

Time, min	Flow rate, mL/min	Mobile phase composition[a] A, %	B, %
0	1.2	60	40
9.0	1.2	36	64
11.0	1.2	10	90
11.5	1.2	60	40
20.0	1.2	60	40

[a]Mobile phase A = acetate buffer (1.5 mol/L, pH = 4.6). Mobile phase B = methanol.

Calculations

A linear external calibration curve, plotting peak area against concentration, was constructed. The concentrations of sorbic acid (mg/kg) in the processed cheese and shredded cheese samples are given by the following equation, which corrects for masses and volumes:

$$\text{Sorbic acid } (\text{mg/kg}) = \frac{A}{L} \times \frac{26.5}{M_s} \times \frac{1}{0.05} \times \frac{1000}{1000}$$

where A = peak area of sorbic acid in the sample; L = slope of the calibration curve of standard peak area versus concentration (μg/mL); 26.5 = final volume of the cheese sample plus methanol (mL); M_S = mass of the sample (g); 1 = final volume of the extract (mL); 0.05 = volume of the aliquot (mL); 1000 = mass conversion factor (μg/g to μg/kg); and 1000 = mass conversion factor (μg/kg to mg/kg).

Results and Discussion

Method Optimization

The major challenge during the development of this method was obtaining homogeneous and representative test samples of the cheese, because of both the propensity of sorbic acid to migrate within the cheese matrix and the irregular dispersal of sorbic acid during application to grated cheese. To overcome homogeneity issues, several different approaches were evaluated.

Initially, multiple random cored subsamples from processed cheese slices were selected, ground with an Ultra-Turrax, and extracted twice into a total of 50 mL of methanol. Analyzing processed cheese slices in this manner was wasteful in solvent, was time consuming, and failed to overcome the homogeneity issue. As the use of larger sample sizes would require significantly more solvent per sample, alternative sampling strategies were investigated.

The heterogeneous dispersal of sorbic acid is even more profound in grated cheese, requiring larger sample sizes to obtain a representative subsample. Initially, 300 g of cheese was suspended in 500 mL of methanol, followed by filtering through a large Buchner funnel. Although this method produced acceptable results, it was not conducive to high-throughput analysis and was exceedingly wasteful in solvent use. A further approach using a Masticator homogenizer with 150 g of cheese and 75 mL of methanol was trialed. This method also produced acceptable precision; however, it was also limited in sample throughput, wasteful in solvent, and vulnerable to leakage.

Subsequently, internal standardization using 4-chlorobenzoic acid was investigated as a possible technique to overcome suboptimal recovery issues; however, this compound did not behave equivalently with sorbic acid during extraction, leading to poor spiked recovery results.

Finally, reducing a large 200 g sample to a granular form using dry ice and taking a 1 g subsample for analysis provided the necessary solution to both the homogeneity issue and the recovery issue, and further precluded the need for an internal standard because of the simplicity of the protocol.

The UV absorption was monitored at 254 nm as this was close to the λ_{max} for sorbic acid, as shown in Figure 1, in contrast to previously published methods that used 235 nm or 280 nm (19, 22). The cheese extracts were diluted in the initial mobile phase, which yielded superior peak shape compared with dilution in methanol. In addition, the pentafluorophenyl column gave shorter retention times for sorbic acid compared with C_{18} reverse phase columns (19, 20, 22). Reversed phase columns with mobile phases containing acetonitrile and acetic acid buffer, or methanol and phosphate buffer have been reported (16, 18, 23).

Method Validation

Detector linearity was determined by the analysis of multi-level sorbic acid standard solutions (n = 8) covering an analyte range of 0.5–38 μg/mL. Linearity was evaluated by least-squares regression analysis of peak area versus concentration, with acceptable values for r^2 of 0.999 being obtained; residual plots were assessed as a further test of linearity, and showed a constant variance with concentration randomly distributed around the line of best fit.

Recovery was determined by spiking both a sliced processed cheese sample and a grated Cheddar cheese sample with sorbic acid to 0, 50, 100, and 200% of a nominal 700 mg/kg. The recovery for sliced cheese ranged from 88.3 to 97.0%, with a mean of

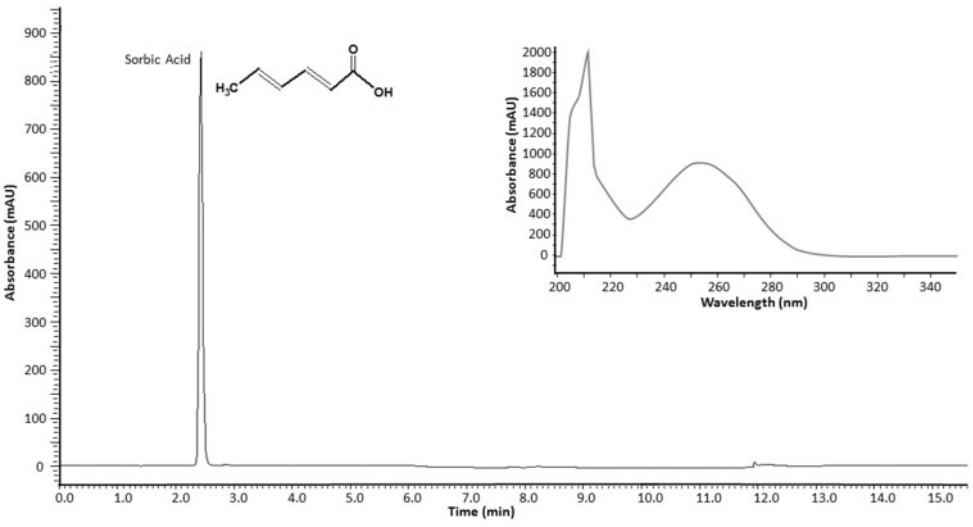

Figure 1. Chromatogram at 254 nm with inset of UV absorbance spectrum of sorbic acid.

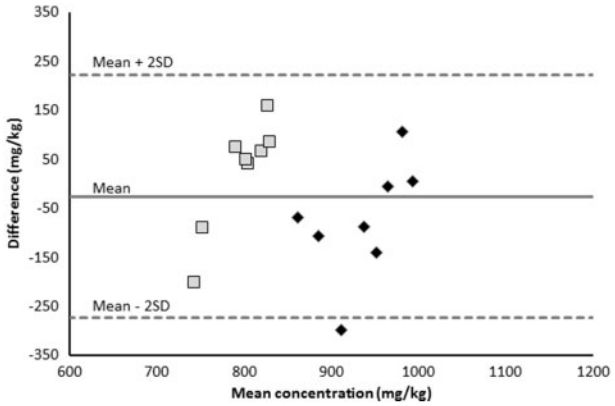

Figure 2. Bland–Altman plot for sorbic acid in cheese, comparing the described LC-UV method with a reference method (21).

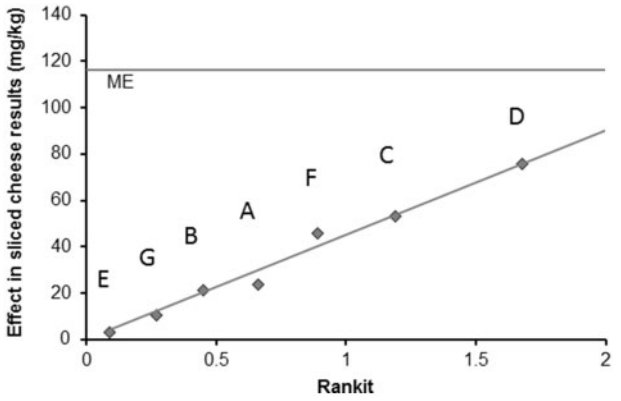

Figure 3. Half-normal plot of method ruggedness evaluation for sliced processed cheese.

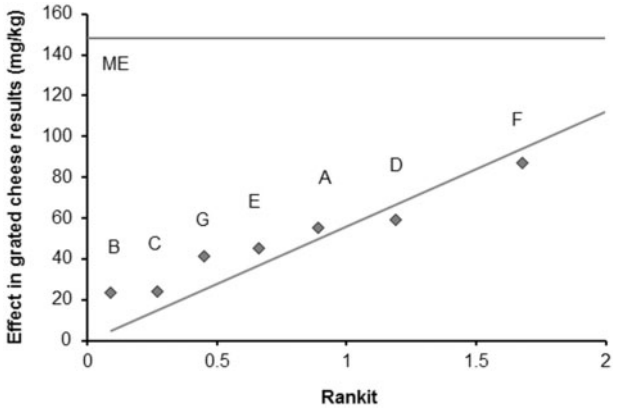

Figure 4. Half-normal plot of method ruggedness evaluation for grated Cheddar cheese.

93.7%, and the recovery for grated cheese ranged from 95.2 to 117.1%, with a mean of 103.7%. The amount of sorbic acid added to processed cheese slices or grated cheese is usually between 600 and 1000 mg/kg to have effective antimicrobial and fungistatic effects, and to comply with EU regulations, although this can be as high as 3000 mg/kg for New Zealand regulations. Due to this wide allowable range, the analytical recovery for this method is acceptable.

An exhaustive extraction ($n = 6$) on sliced processed cheese and grated Cheddar cheese was performed to evaluate the sorbic acid recovered through a single extraction. Recoveries of 95.2 and 94.0% were obtained in the first extraction, demonstrating acceptable recovery without the need for further extraction. By the second extraction, the recovery had reached over 99%.

The method was compared with the International Dairy Federation reference method for the analysis of benzoic acid and sorbic acid in yogurt and cheese (21). Eight replicates each of sliced processed cheese and grated Cheddar cheese samples were tested by each method and a paired *t*-test was used to evaluate bias. The bias between the measured results obtained by the described method and the reference method was not statistically significant (mean bias: processed cheese = 23 mg/kg, $p = 0.59$; grated Cheddar cheese = 74 mg/kg, $p = 0.13$). Bland–Altman plots illustrate the agreement between the analytical methods (Figure 2).

Method precision was evaluated by the analysis of duplicate pairs of sliced processed cheese and grated Cheddar cheese samples. Acceptable precision was demonstrated, with a repeatability of 5.3 and 4.3% relative standard deviation (RSD) and an intermediate precision of 5.3 and 6.9% RSD (HorRat: 1.3, 1.7) for sliced processed cheese and grated Cheddar cheese, respectively (28).

The limit of detection (LOD) was estimated by serial dilution of a sorbic acid standard until a signal-to-noise ratio of three was obtained (29). The measured value for the LOD was 0.05 μg/mL on-column, which equates to approximately 2 mg/kg, signifying that the described method is suitable for application to manufactured cheese products, as these typically contain sorbic acid at concentrations at least two orders of magnitude higher. The instrument LOQ for this method is 0.167 μg/mL which corresponds to 6.7 mg/kg of sorbic acid in the cheese.

The robustness of the method was assessed by conducting a Plackett–Burman trial (30, 31) in the manner described previously (32). The seven factors assessed were: sample weight (1.2 and 0.8 g) (A, a), volume of methanol (28 and 22 mL) (B, b), Ultra-Turrax time (90 and 30 s) (C, c), centrifuge speed (1800 and 1400 × *g*) (D, d), centrifuge time (12 and 8 min) (E, e), extraction volume (55 and 45 μL) (F, f), and a dummy factor (G, g). The method was found to be robust for the parameters evaluated, and the results obtained were normally distributed, with variances conforming to that expected by chance (Figures 3 and 4). As with similar external standard-based methods, critical method parameters included accurate measurement of the sample weight, extract volume, and aliquot volume.

The method described can yield a single result in approximately 1 h, with the procedure being capable of significant sample throughput, with more than 40 samples per day completed by a single analyst.

Conclusions

A facile method that is intended for use in high-throughput laboratories as part of routine product compliance release testing of sorbic acid in manufactured cheese products is described. The initial sample preparation incorporates the physical grinding of the cheese in combination with dry ice, followed by further size reduction with an Ultra-Turrax, a procedure that has facilitated the methanolic extraction of homogeneous and representative test samples before quantitative analysis by HPLC–UV. This method was subjected to single-laboratory validation and was found to be accurate, precise, and fit-for-purpose.

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