Table P4 Results of quantitative analysis by qNMR and GC-FID

No.	Compound	Content (%)		Ratio of isomers		
		qNMR	GC-FID	Main / Minor	qNMR	GC-FID
P1	2-Ethyl-6-methylpyrazine	98.5[a)]	99.7[a)]	2-Ethyl-6-methylpyrazine / 2-Ethyl-5-methylpyrazine	69.8 / 28.7	70.3 / 29.4
P2	2-Ethyl-5-methylpyrazine	98.8[a)]	99.3[a)]	2-Ethyl-6-methylpyrazine / 2-Ethyl-5-methylpyrazine	57.6 / 41.3	57.7 / 41.5
P3	2-Ethyl-3-methylpyrazine	98.9	99.9			
P4	Pyrazine	99.0	-[b)]			
P5	2-Methylpyrazine	98.7	100.0			
P6	2-Ethylpyrazine	98.9	99.7			
P7	2,3-Dimethylpyrazine	99.4	99.6			
P8	2,5-Dimethylpyrazine	97.2	99.9			
P9	2,6-Dimethylpyrazine	99.1	99.8			
P10	2,3-Diethylpyrazine	98.8	98.7			
P11	2-Ethyl-3,(5 or 6)-dimethylpyrazine	98.4[a)]	98.7[a)]	2-Ethyl-3,5-dimethylpyrazine / 2-Ethyl-3,6-dimethylpyrazine	53.0 / 45.4	54.4 / 44.3
P12	2.3.5-Trimethylpyrazine	99.8	99.9			
P13	5,6,7,8-Tetrahydroquinoxaline	98.4	99.1			
P14	6,7-Dihydro-5-methyl-5*H*-cyclopentapyrazine	98.1	98.7			

a) Sum of isomers. b) Not measured.

Table D1 Instruments and acquisition parameters

Spectrometer	ECA600 (JEOL)
Probe	5 mm broadband autotune probe
Spectral width	- 5 - 15 ppm
Data points	32000
Auto filter	on (8 times)
Flip angle	90°
Pulse delay	60 s (>5*T_1)
Scan times	8
Sample spin	no spin
Probe temperature	25°C
Solvent	D_2O
qNMR reference material	DSS-d_6
Primary standard material	Potassium hydrogen phthalate (PHP) (NMIJ CRM3001a)

Table D2 The Purity of Tar Dyes calcd. by qNMR.

Sample		Purity (%)	Target signals for qNMR / Number of the Protons									RSD(%) in molecule (n = Target Signals)
			7.42	7.62	7.77	7.88	8.05	8.16	8.26-8.38			
			2H	1H	1H	1H	1H	2H	2H			
R2	AV(%)	84.9	84.3	85.0	84.0	84.4	85.6	85.7	85.3			0.8
	RSD(%) in sample (n =3)	0.3	0.5	0.4	0.7	0.2	0.7	0.5	0.2			
			7.00	7.58	7.65	7.71	7.79					
			1H	1H	1H	2H	1H					
R3	AV(%)	89.4	84.1	90.3	94.8	87.5	90.2					4.1
	RSD(%) in sample (n =3)	1.8	2.3	2.2	2.0	1.8	1.1					
			2.34	3.50	6.02	6.95	7.11	7.64	7.70	7.87		
			3H	3H	1H	2H	1H	1H	1H	1H		
R40	AV(%)	83.5	84.3	84.6	80.6	82.7	84.7	85.2	85.0	81.0		2.1
	RSD(%) in sample (n =3)	0.5	0.5	0.6	0.5	0.6	0.8	0.3	0.5	0.6		
			6.51	7.44	7.52	7.58	7.86	7.99	8.28	8.75	8.92	
			1H	1H	1H	1H	1H	1H	1H	1H	1H	
R102	AV(%)	83.9	82.2	84.2	83.7	85.2	83.5	83.9	83.7	84.7	83.7	1.1
	RSD(%) in sample (n =3)	0.2	0.5	1.3	0.7	0.6	0.3	0.3	0.3	0.5	0.2	
			7.45									
			2H									
R104	AV(%)	85.5	85.5									-
	RSD(%) in sample (n =3)	0.3	0.3									
			7.67									
			2H									
R105	AV(%)	82.5	82.5									-
	RSD(%) in sample (n =3)	0.2	0.2									
			1.19	3.53	6.63	6.82	6.96	7.61	8.24	8.50		
			12H	8H	2H	2H	2H	1H	1H	1H		
R106	AV(%)	92.3	95.0	95.7	92.9	93.6	93.1	89.4	89.5	88.9		2.7
	RSD(%) in sample (n =3)	1.4	1.1	1.3	1.5	1.4	1.8	1.7	1.7	1.0		
			7.66	7.85-7.95	7.97							
			2H	4H	2H							
Y4	AV(%)	85.7	80.9	88.6	87.8							4.3
	RSD(%) in sample (n =3)	1.2	1.6	1.2	0.9							
			6.37	7.15-7.50	7.60-7.80	8.00						
			1H	3H	4H	1H						
Y5	AV(%)	86.2	83.9	88.1	88.3	84.4						2.4
	RSD(%) in sample (n =3)	1.4	1.7	1.1	1.3	1.6						
			1.23	3.65	7.02	7.30-7.80	8.02					
			6H	4H	2H	5H	1H					
B1	AV(%)	84.2	83.8	82.2	83.0	87.9	84.1					2.6
	RSD(%) in sample (n =3)	4.1	3.1	2.9	5.8	3.3	5.4					
			6.60	7.60	7.94							
			2H	2H	2H							
B2	AV(%)	89.2	89.3	89.6	88.8							0.4
	RSD(%) in sample (n =3)	1.2	1.4	1.2	1.2							
			1.23	3.64	7.05	7.20-7.32	7.51	7.60-7.78				
			6H	4H	2H	2H	1H	4H				
G3	AV(%)	87.6	94.6	69.3	91.4	83.9	93.3	93.1				10.3
	RSD(%) in sample (n =3)	1.0	1.0	0.8	1.1	0.8	1.8	1.1				

Table D3 Summary of tar dyes purities calcd. by TiCl3 titration, gravimetry, spectrophotometry and qNMR.

	qNMR (n =3)		$TiCl_3$ titration* (n = 3)		Gravimetry* (n = 4)		Spectrophotometry (n = 5)		Official method/qNMR
	AV(%)	RSD(%)	AV(%)	RSD(%)	AV(%)	RSD(%)	AV(%)	RSD(%)	
R2	84.9	0.3	91.8	1.7			93.2	3.4	1.08
R3	89.4	1.8			94.5	1.1	94.6	3.0	1.06
R40	83.5	0.5	93.7	0.3			93.9	5.2	1.12
R102	83.9	0.2	92.3	1.3			93.4	4.2	1.10
R104	85.5	0.3			93.1	0.4	91.2	1.8	1.09
R105	82.5	0.2			92.3	0.7	91.8	1.4	1.12
R106	92.3	1.4	96.5	2.0			95.4	1.2	1.05
Y4	85.7	1.2	92.5	1.2			92.0	1.7	1.08
Y5	86.2	1.4	93.6	0.9			92.2	1.8	1.09
B1	84.2	4.1	91.7	2.1			88.8	1.5	1.09
B2	89.2	1.2	90.9	1.9			95.3	1.8	1.02
G3	87.6	1.0	88.8	1.5			91.7	2.1	1.01

* Official method for purity test.

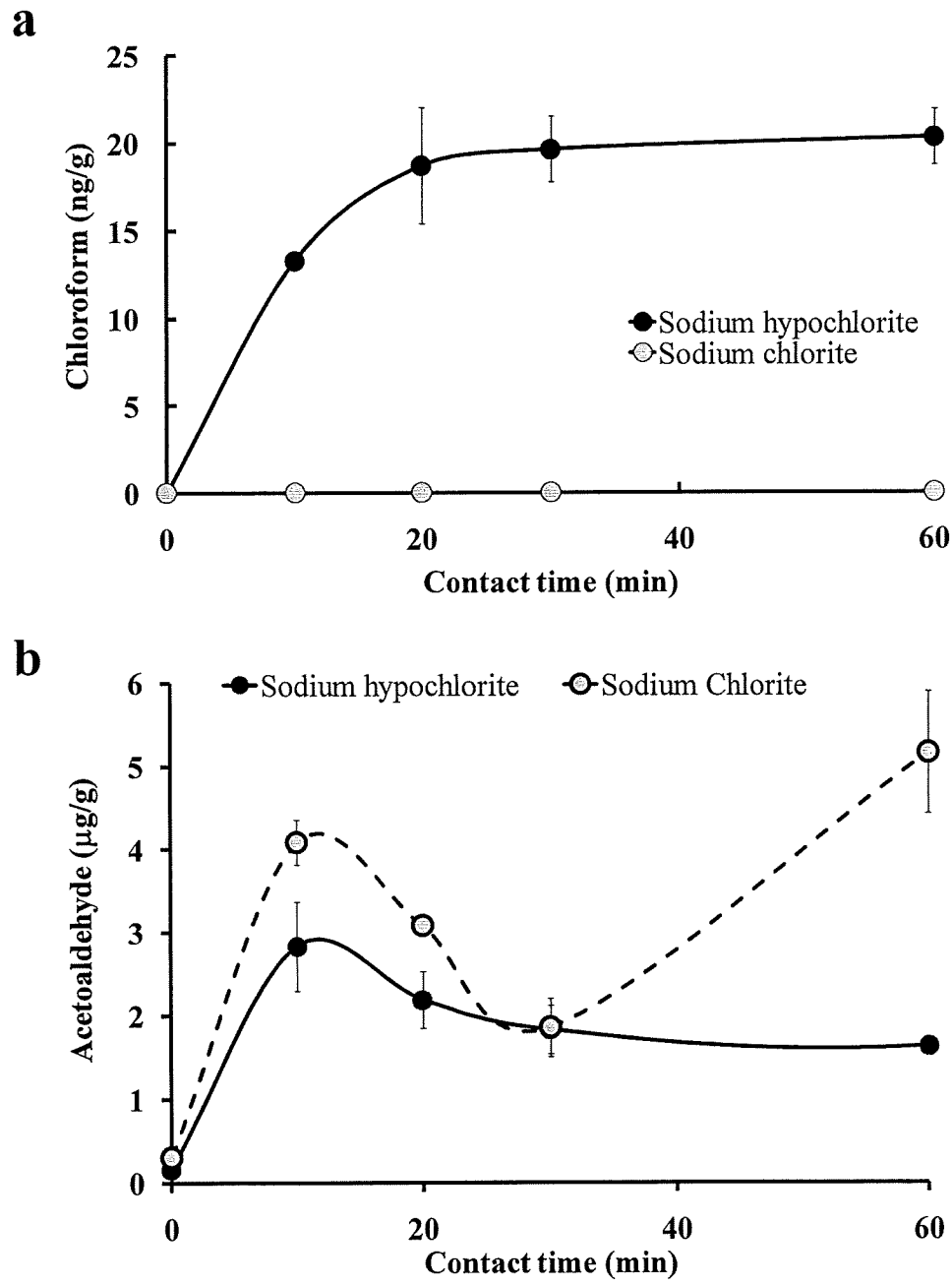

Fig.1. 次亜塩素酸ナトリウム及び亜塩素酸ナトリウム処理カット野菜中における消毒副生成物生成量の経時変化. a)クロロホルム量の推移, b)アセトアルデヒド量の推移

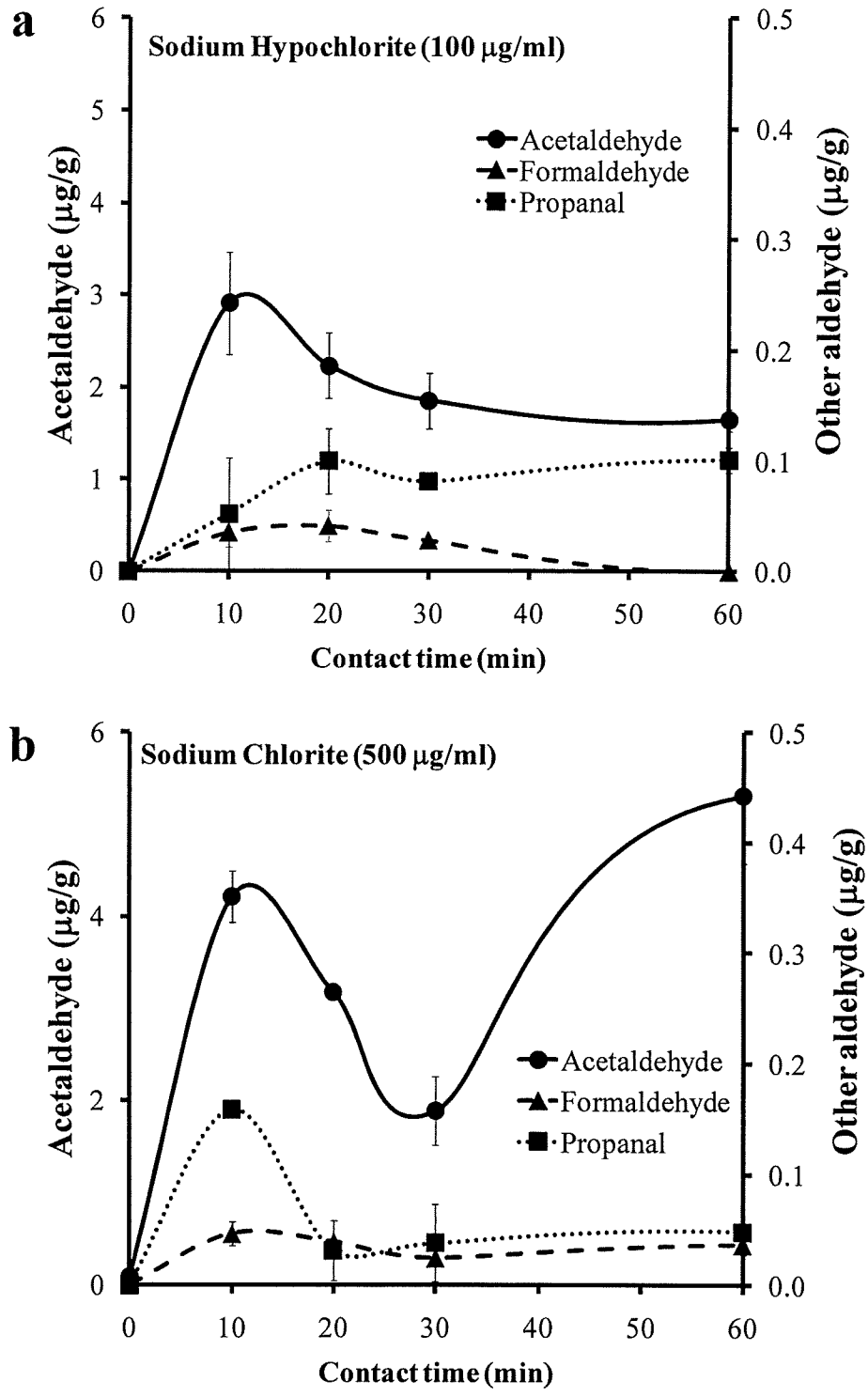

Fig. 2. 次亜塩素酸ナトリウム及び亜塩素酸ナトリウム処理カット野菜中におけるアルデヒド類生成量の経時変化.

a) 次亜塩素酸ナトリウム処理試料, b) 亜塩素酸ナトリウム処理試料

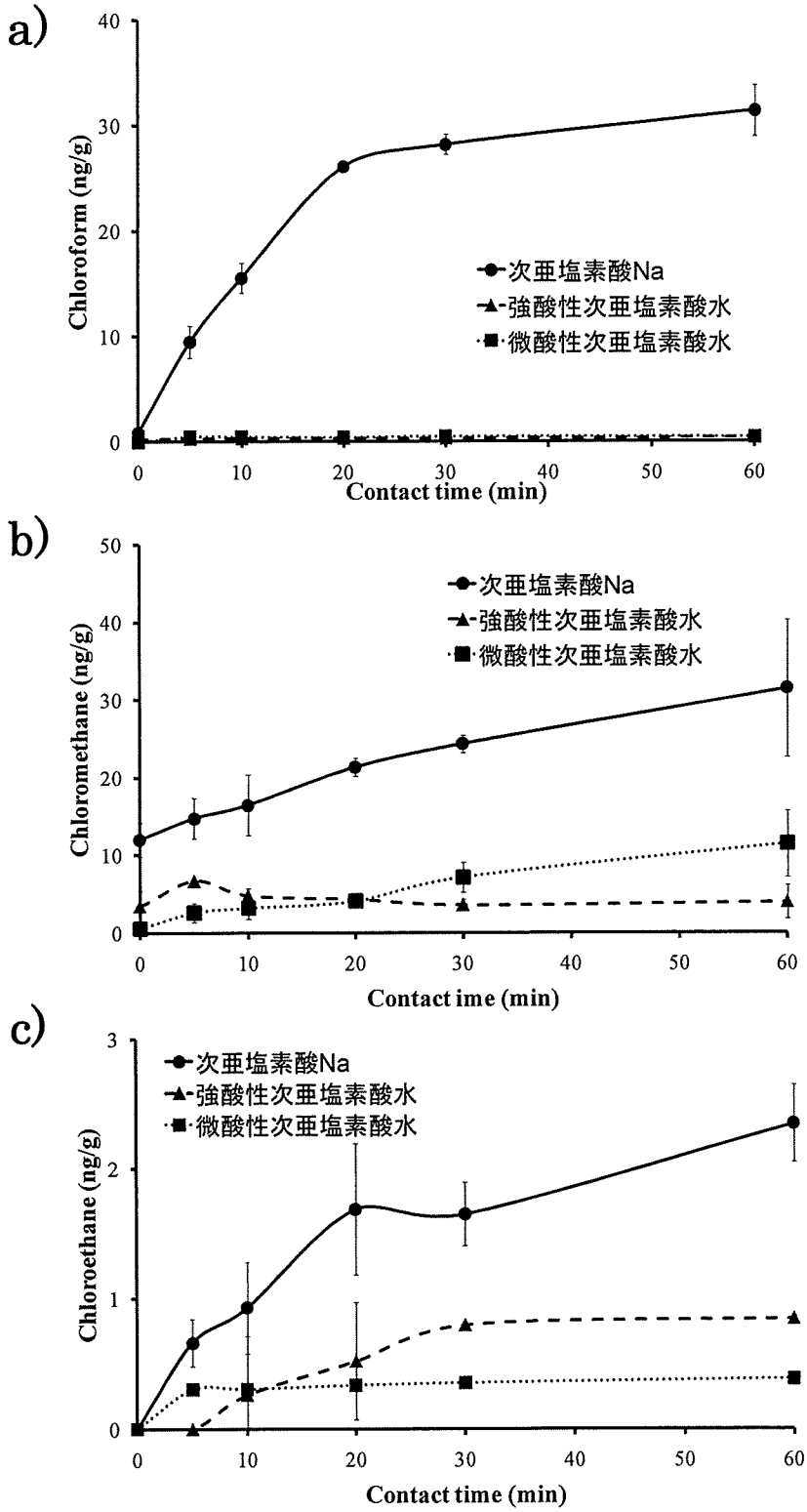

Fig. 3.　各種塩素系殺菌料での殺菌処理によるカット野菜中の各消毒副生成物量の経時変化．ａ）クロロホルム量の推移、ｂ）クロロメタン量の推移、ｃ）クロロエタン量の推移

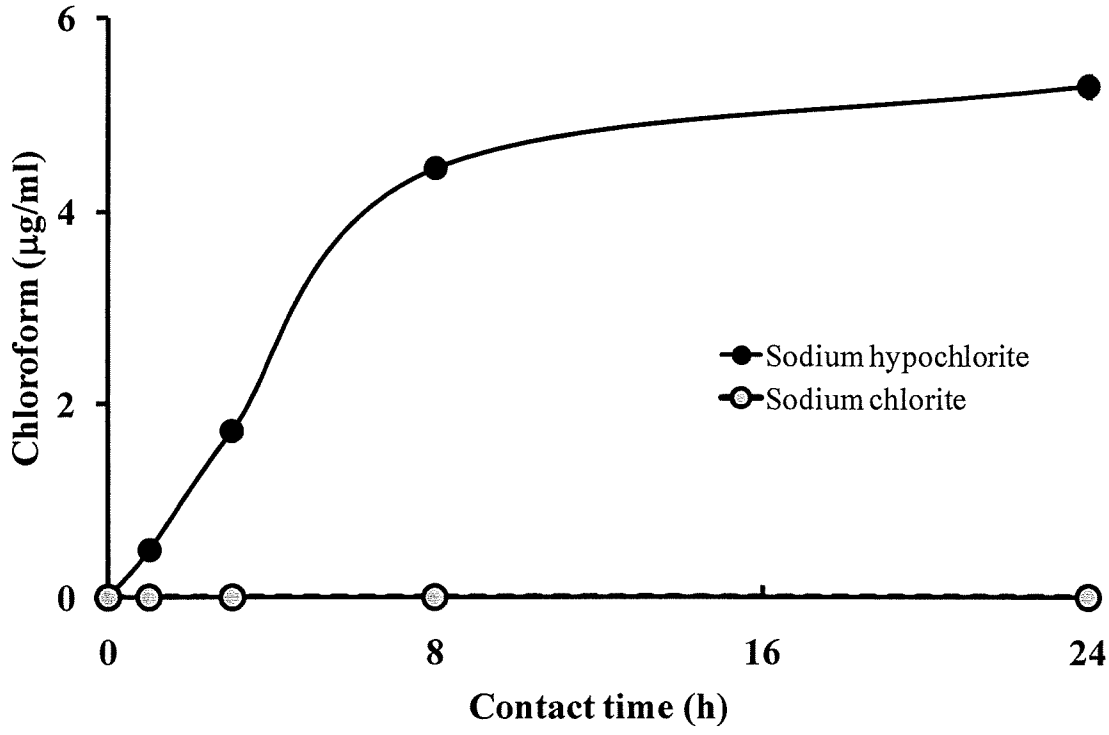

Fig. 4. 殺菌液へのクエン酸混和によるクロロホルム生成量の推移

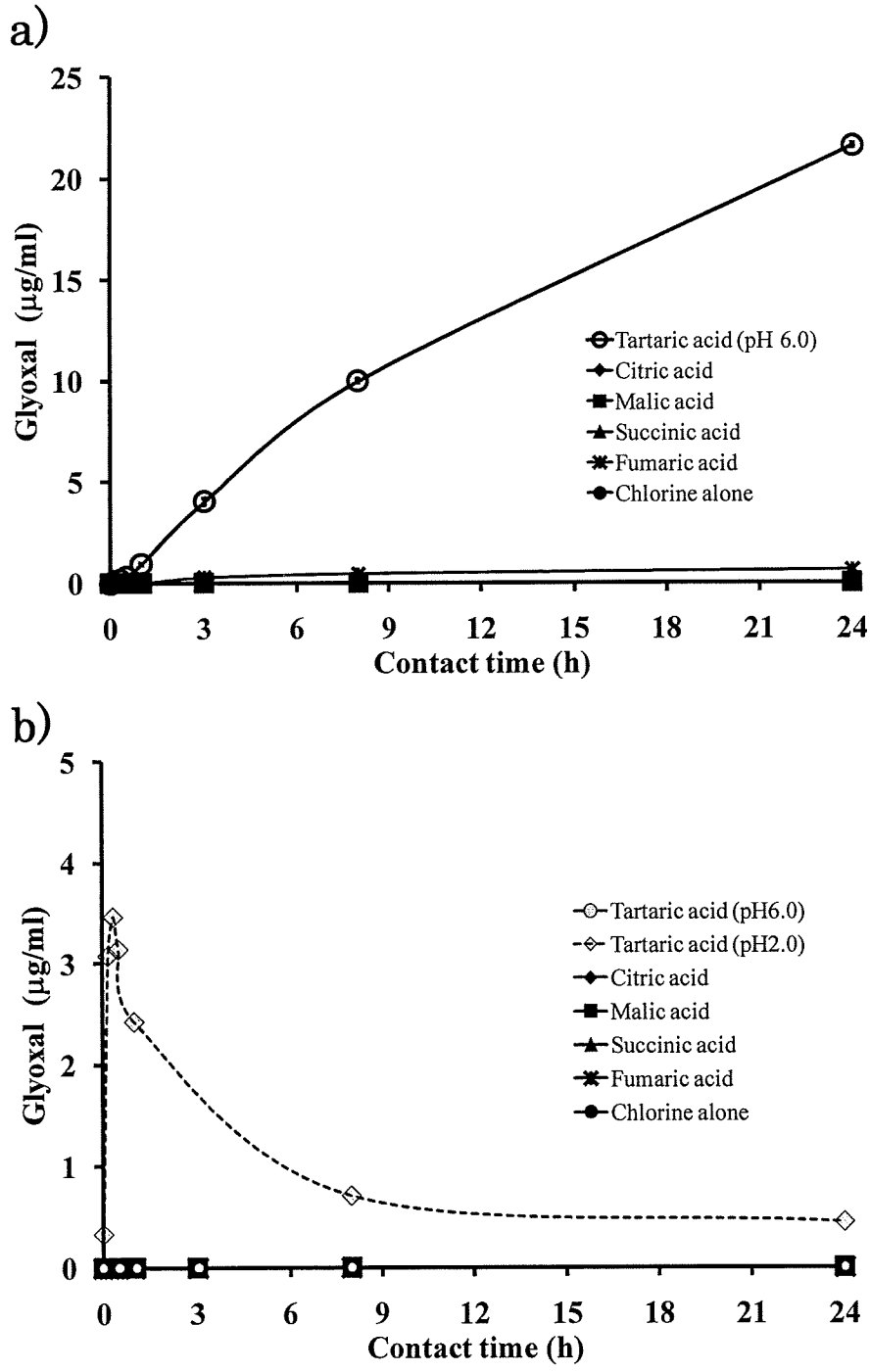

Fig. 5. 殺菌液への有機酸混和によるアルデヒド類生成量の推移
a）次亜塩素酸ナトリウム・有機酸混液中のアルデヒド量の推移, b）亜塩素酸ナトリウム・有機酸混液中のアルデヒド量の推移

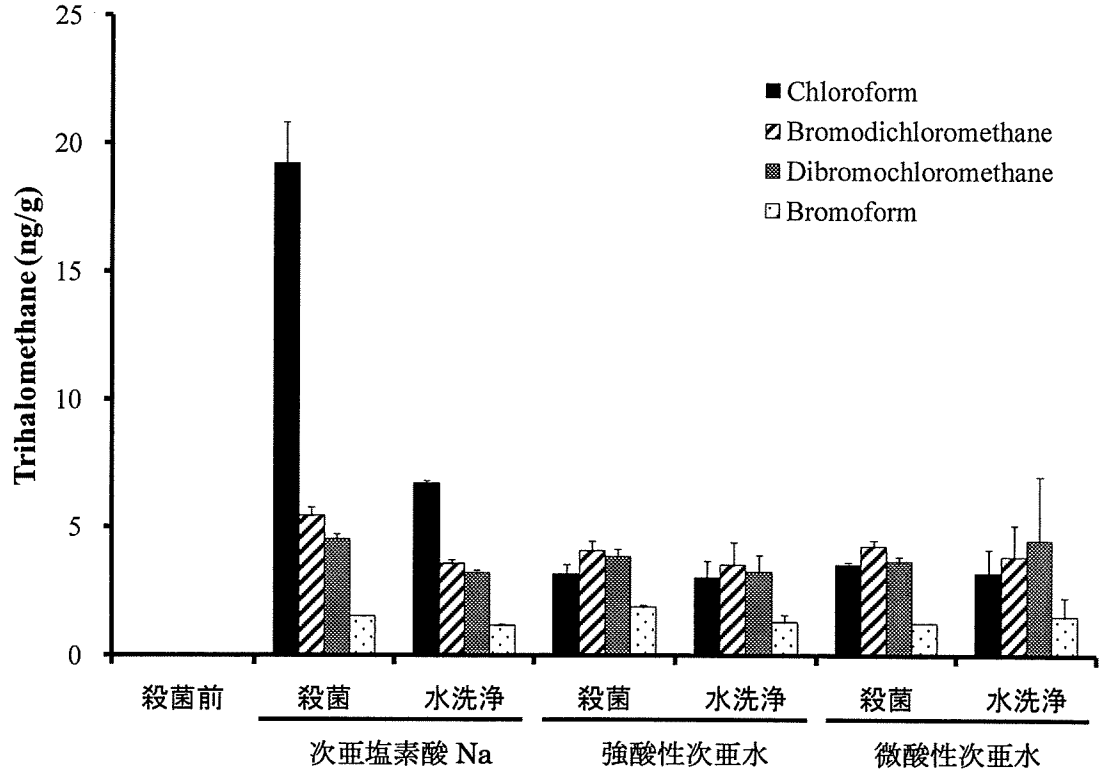

Fig. 6. 各種殺菌料によるカット野菜の殺菌・洗浄処理後の THM 残存量の推移

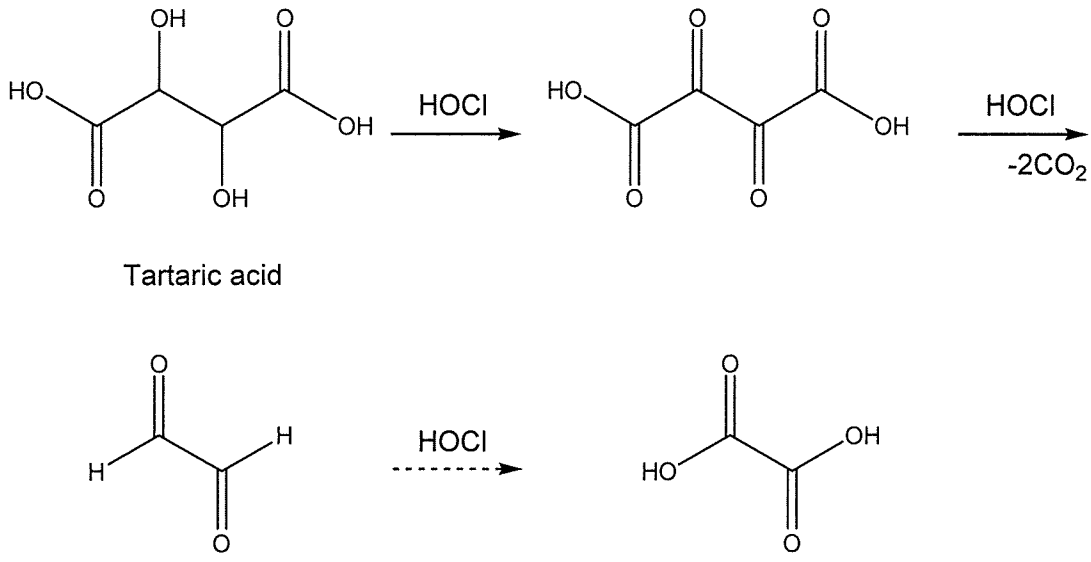

Fig.7. 酒石酸の次亜塩素酸ナトリウム及び亜塩素酸ナトリウムとの反応による
グリオキサール生成メカニズム（仮説）

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Sugimoto, N., Koike, R., Furusho, N., Tanno, M., Yomota, C., Sato, K., Yamazaki, T., Tanamoto, K.	Quantitative nuclear magnetic resonance spectroscopic determination of the oxyethylene group contents of polysorbates	Food Add. Contam.	24(8)	799-806	2007
Mine, T., Okada, Y., Semma, M.	The interaction of sorbic acid with amino acid may alter the quality of processed foods somewhere in the food chain from production to table	Japanese Journal of Food Chemistry and Safety	14(1)	23-26	2007
Horiyama, S., Honda, C., Suwa, K., Umemoto, Y., Okada, Y., Semma, M., Ichikawa, A., Takayama, M.	Sensitive and simple analysis of sorbic acid using liquid chromatography with electrospray ionization tandem mass spectrometry	Chem. Pharm. Bull.	56(4)	578-581	2008
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Quantitative nuclear magnetic resonance spectroscopic determination of the oxyethylene group content of polysorbates

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Abstract
Guidelines for the oxyethylene group (EO) content of polysorbates are set by the Food and Agriculture Organization/World Health Organization Joint Expert Committee on Food Additives. However, the classical titration method for EO determination is difficult and time-consuming. Here, we show that quantitative ^{1}H-nuclear magnetic resonance spectroscopy can determine the EO contents of polysorbates rapidly and simply. The EO signals were identified through comparisons with sorbitan monolaurate and poly(ethylene glycol) distearate. Potassium hydrogen phthalate was used as an internal standard. The EO contents were estimated from the ratio of the signal intensities of EO to the internal standard. Two nuclear magnetic resonance systems were used to validate the proposed method. The EO content of commercial polysorbates 20, 60, 65, and 80 was determined to be within the recommended limits using this technique. Our approach thus represents an additional or alternative method of determining the EO contents of polysorbates.

Keywords: *Analytical method, food additive, oxyethylene, polysorbate, quantitative nuclear magnetic resonance*

Introduction

Polysorbates are non-ionic surfactants that are widely used as emulsifiers, dispersants, and stabilizers in food processing. Polysorbates consist of a mixture of fatty-acid partial esters of sorbitol and condensed sorbitol anhydrides, and contain approximately 20 moles of ethylene oxide (comprising the oxyethlene unit [EO] $-OC_2H_4-$) for each mole of sorbitol, along with its monohydrides and dianhydrides. The main fatty acids of polysorbates 20, 60, 65, and 80 are monolauric acid, monostearic acid, tristearic acid, and monooleic acid, respectively. The typical structures of these polysorbates are shown in Figure 1.

Guidelines for the EO contents of polysorbates are set by the Food and Agriculture Organization (FAO)/World Health Organization (WHO) Joint Expert Committee on Food Additives (JECFA). To comply with the JECFA standards, the quality and composition of commercially synthesized polysorbates must be monitored and regulated. The standard method of measuring EO as described in "section VI. Methods for fats and related substances in the guide to specification" is as follows: "The oxyethylene groups are converted to ethylene and ethyl iodide which can be determined by titration. By utilizing a conversion factor determined on a reference sample, it is possible to compute the polyoxyethylene ester content" (JECFA [internet]). However, this classical titration method requires a complicated apparatus and involves several time-consuming steps. Alternative methods for determining the EO contents of polysorbates have not previously been reported, because these complex compounds are mixtures of isomers that are non-selectively substituted with EOs and fatty acids.

The quantitative nuclear magnetic resonance (qNMR) approach is based upon the International

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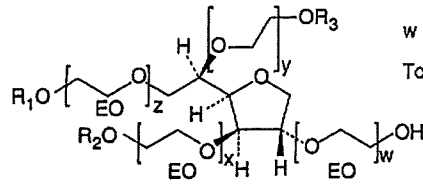


w + x + y + z = approx. 20

Total EO = (C_2H_4O) x (w + x + y + z) = $C_{40}H_{80}O_{20}$ (MW 881.1)

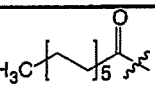
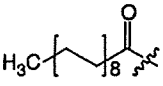
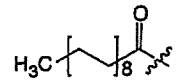
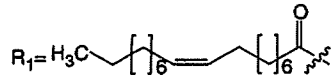
Compound			Formula (MW)	EO(%) in molecule
Polysorbate 20 (polyoxyethylene (20) sorbitan monolaurate)	R_1= 	$R_2 = R_3 = H$	$C_{58}H_{114}O_{26}$ (MW1227.5)	EO(%) = 71.8
Polysorbate 60 (polyoxyethylene (20) sorbitan monostearate)	R_1= 	$R_2 = R_3 = H$	$C_{64}H_{126}O_{26}$ (MW1311.7)	EO(%) = 67.2
Polysorbate 65 (polyoxyethylene (20) sorbitan tristearate)	$R_1 = R_2 = R_3$ = 		$C_{100}H_{194}O_{28}$ (MW1844.6)	EO(%) = 47.8
Polysorbate 80 (polyoxyethylene (20) sorbitan monooleate)	R_1= 	$R_2 = R_3 = H$	$C_{63}H_{122}O_{26}$ (MW1295.6)	EO(%) = 68.0

Figure 1. Typical structures of polysorbates 20, 60, 65, and 80. The formulae and EO (%) were estimated based on the assumption that there were 20 moles of EO per molecule.

system of units (SI units). This valuable technique meets the requirements of a primary ratio analytical method (Jancke 1998). The use of qNMR to determine the ethanol content of deuterium oxide solution was previously reported as a part of an intercomparison study organized by the Comité Consultatif pour la Quantité de Matère (CCQM). The results showed that the accuracy of qNMR was equivalent to that of gas chromatography with a flame ionization detector (GC-FID) (Saito et al. 2003). qNMR exploits the fact that the signal intensities of a given NMR resonance are directly proportional to the molar amount of the nucleus within the sample. qNMR can determine the quantity of a compound, its substituent contents, or its absolute quality if the whole sample weight is known. This technique has several advantages for the analysis of organic compounds: it is non-destructive, it provides both quantitative data and structural information about a compound, and high-throughput spectral-acquisition instruments are commercially available. The main drawback of the qNMR approach is that manual spectral assignment is required; however, this can easily be rectified by applying current NMR technical experiments such as total correlated spectroscopy (TOCSY), heteronuclear multiple quantum correlation (HMQC), heteronuclear multiple bond coherence (HMBC), etc.

Based on these features of qNMR, we predicted that the method could be used to determine the EO contents of polysorbates. In the current paper, we detail the application of qNMR along with an internal standard for the direct determination of the EO contents of polysorbates.

Materials and methods

Materials

Samples of reagent-grade polysorbates 20, 60, 65, 80, and sorbitan monolaurate (Span 20) were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Poly(ethylene glycol) distearate was purchased from Sigma-Aldrich Japan KK (Tokyo). Commercial samples of polysorbates were obtained from companies A–E via the Japan Food Additives Association. The NMR solvents, methanol-d_4 and acetone-d_6 with 0.03% tetramethylsilane (TMS), were purchased from Isotec Inc. (Miamisburg, OH). Potassium hydrogen phthalate (PHP), which was standard grade for volumetric analysis according to Japanese Industrial Standard (JIS) K8005, was purchased from Wako Pure Chemical Industries, Ltd.

Instrumentation

NMR spectra were recorded on JNM-ECA (500 MHz; JEOL, Tokyo) and MERCURY (400 MHz; VARIAN, Palo Alto, CA) pulsed Fourier-transform (FT) spectrometers, equipped with 5 mm $^1H\{X\}$ inverse detection gradient

Table I. Instruments and acquisition parameters.

Spectrometer	MERCURY400 (VARIAN) and ECA500 (JEOL)
Probe	5 mm indirect detection probe
Spectral width	2.5–12.5 ppm
Data points	64 000
Flip angle	45°
Pulse delay	30 s (>5 * T_1)
Scan times	8
Sample spin	15 Hz
Probe temperature	25°C
Solvent	Mixture of methanol-d_4 and acetone-d_6 (1:1)
Internal standard	Potassium hydrogen phthalate (PHP)
Range of integral signal	Oxyethylene group (EO) = 3.40–3.85 ppm 4 protons of PHP = 7.46–7.66 ppm + 8.18–8.38 ppm

probes, with methanol-d_4:acetone-d_6 (1:1) and 0.3% (w/v) PHP as an NMR solvent. The spectra were referenced internally to TMS by ^{1}H-NMR. The samples and internal standard were weighed on a LIBROR AEG-80SM (Shimadzu, Kyoto, Japan) electronic balance to an accuracy of ±0.01 mg.

Preparation of samples and NMR measurement conditions

The polysorbate samples were prepared as follows. PHP was crushed into a powder in a mortar and dried for 1 h at 120°C. After cooling in a desiccator, the powder (300 mg) was dissolved in 100 ml of methanol-d_4:acetone-d_6 (1:1) with ultrasonic agitation for 30 min. This stock solution was used as the NMR solvent and included an internal standard. A 50-mg polysorbate sample was then dissolved in 3 ml of the NMR solvent described above, and 0.6 ml of the sample solution was placed into a 5-mm NMR tube (Kusano Science Co. Ltd, Tokyo). The ^{1}H-NMR spectra were recorded on MERCURY400 and ECA500 spectrometers operating at 400 and 500 MHz, respectively. Typical ^{1}H-NMR parameters for the quantitative analyses are listed in Table I. The free induction decay (FID) signals of the samples from the MERCURY400 and ECA500 spectrometers were loaded onto a Windows XP-based personal computer (PC) equipped with the Alice 2 Version 5 (JEOL) NMR data-processing and analytical software. Fourier transformations of the FID signals were carried out with this software using the default parameters; window function = exponential, BF = 0.12 Hz, zero filling = 1, T1 = T2 = 0%, T3 = 90%, T4 = 100%. After phase adjustments and baseline corrections of the NMR spectra were performed using the same algorithms in the automatic mode of Alice 2, the signal intensities of the EOs and internal standard protons were measured, respectively.

Results and discussion

Identification of EO signals in polysorbates

Polysorbate molecules contain approximately 20 moles of EO according to the JECFA definition. However, recently reported matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) spectra showed that polysorbates include numerous other chemical species, including polyethylenes, unesterified, monoesterified, and diesterified polyoxyethylene sorbitans, and isosorbides (Frison-Norrie and Sporns 2001). Furthermore, analysis by liquid chromatography (LC)–mass spectrometry (MS) confirmed that polysorbates contain not only polyoxyethylene sorbitan fatty acid esters but also numerous intermediates, such as polyoxyethylene sorbitan and isosorbitan, and the monoesters and diesters of fatty acids (Vu Dang et al. 2006). These studies have confirmed that polysorbates comprise many types of chemical isomers. This molecular diversity makes it difficult to determine the EO contents of polysorbates. However, we hypothesized that the EO contents of polysorbates could be measured rapidly and simply by qNMR if the signals could be identified on ^{1}H-NMR spectra, regardless of whether they contained numerous chemical isomers.

Thus, in order to identify the EO signals in polysorbates, we compared the ^{1}H-NMR spectra of polysorbate 20, sorbitan monolaurate, and poly(ethylene glycol) distearate. The partial structures of sorbitan monolaurate and poly(ethylene glycol) distearate, which comprised a sorbitol anhydride core and poly(ethylene glycol), were similar to those of polysorbate 20 (Figures 2 and 3). The sorbitan monolaurate and poly(ethylene glycol) distearate spectra revealed fatty-acid moiety signals with δ_H values ranging from 0.9 to 2.4 ppm, similar to those of polysorbate 20. The triplet signal at $\delta_H c.$ 0.9 ppm, the major broad signal and multiplet signal at $\delta_H c.$ 1.3 ppm and 1.6 ppm, and the triplet signal at $\delta_H c.$ 2.4 ppm were identified as the terminal CH_3–, $-CH_2-$, and $-CH_2C{=}O-$ groups of the fatty acids, respectively. Most of the EO signals in poly(ethylene glycol) distearate were observed between δ_H values of 3.40 and 3.85 ppm. One of the $-CH_2O-$ groups appeared to have been shifted downfield to $\delta_H c.$ 4.2 ppm, near to the residual proton of methanol-d_4 at $\delta_H c.$ 4.4 ppm. A HMBC experiment revealed that the proton at $\delta_H c.$ 4.2 ppm was correlated to the carbonyl carbon of the fatty acid at δ_C 173.4 ppm. Thus, the proton signal was assigned

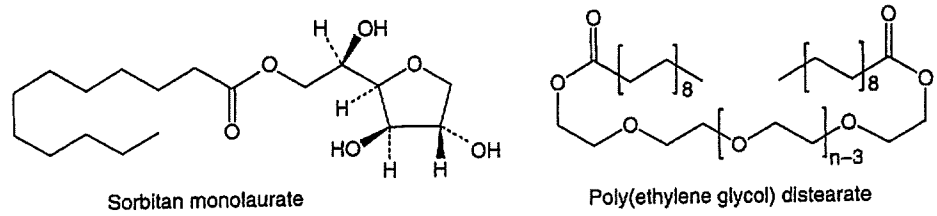


Figure 2. Structures of sorbitan monolaurate and poly(ethylene glycol) distearate.

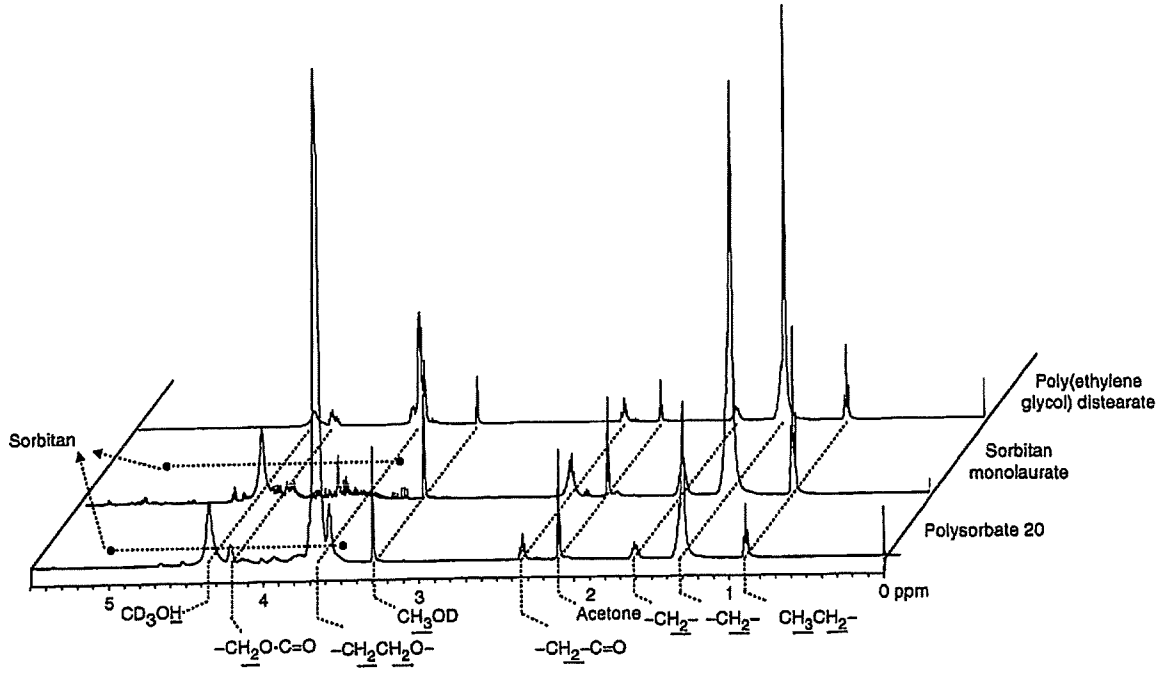


Figure 3. Comparison of NMR spectra of polysorbate 20, sorbitan monolaurate and poly(ethylene glycol) distearate. ^{1}H-NMR spectra were obtained using the ECA500 system (500 MHz; JEOL) under the conditions shown in Table I.

to the $-CH_2O-$ group adjacent to the fatty acid side chain. In the sorbitan monolaurate spectrum, various minor proton signals were observed from δ_H values of *c.* 3.4–5.0 ppm; these were attributed to the sorbitan moiety in sorbitan monolaurate, which consists of a mixture of cyclic sorbitol-derived ethers (such as sorbitan, isosorbite, and other isomers). These signals were also observed on the spectrum of polysorbate 20. However, the signals were broad and negligibly smaller than that of sorbitan monolaurate, as polysorbate 20 has the diversity of molecule more than sorbitan monolaurate. The polysorbate 20 signals ranging from δ_H 0.9 to 2.4 ppm that were attributed to the fatty-acid moiety were similar to those of sorbitan monostearate and poly(ethylene glycol) distearate. Polysorbate 60, 65, and 80 also showed the signals of fatty acid as same as sorbitan monolaurate, but the olefinic protons were only observed at δ_H 5.3 ppm on the spectrum of polysorbate 80 consisting of an unsaturated fatty acid (data not shown). The EO signals were assigned to a large envelop between δ_H 3.40 and 3.85 ppm, and at δ_H 4.20 ppm, which overlapped with the negligible small broad signals seen for the mixture of sorbitan, isosorbite, and other isomers moieties between δ_H values of *c.* 3.4 and 5.0 ppm. The EO signals of polysorbates 60, 65, and 80 also appeared within these ranges (data not shown). This was due to the fact that polysorbates basically comprise the same units: sorbitol anhydrides core, EO chains, and fatty acids. Although proton signals of the $-CH_2O-$ group adjacent to the fatty acid at δ_H *c.* 4.20 ppm were observed, and the signals of the sorbitol anhydrides core were overlapped on EO signals, they were negligible and did not effect the

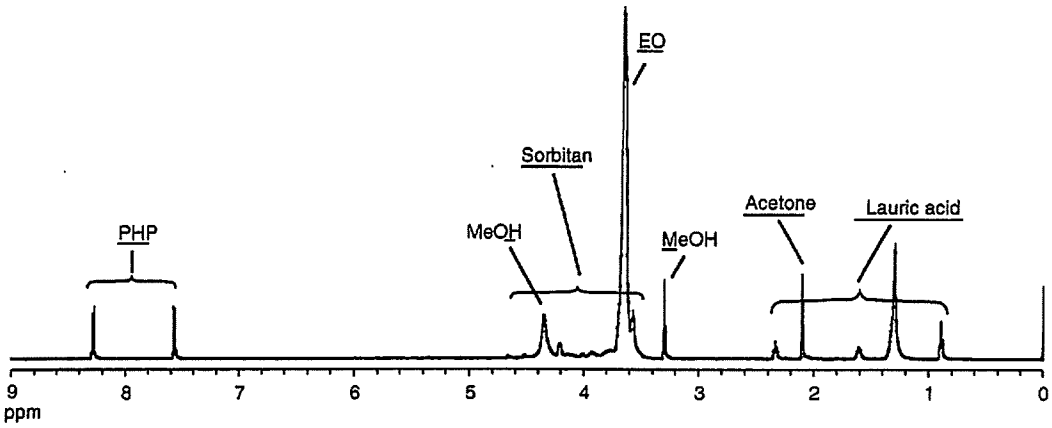


Figure 4. ^{1}H-NMR spectrum of polysorbate 20. The spectrum was obtained using the ECA500 system (500 MHz; JEOL). PHP was added as an internal standard. Signals of the four protons on the benzene ring of PHP were observed at δ_H values of 7.46–7.66 ppm and 8.18–8.38 ppm. Most of the EO signals of polysorbate 20 were observed in a large envelope between δ_H 3.40 and 3.85 ppm.

determination of the EO contents. Thus, in the current research, we used the EO signals between δ_H 3.40 and 3.85 ppm to determine the EO contents of polysorbates by NMR.

Determination of EO contents in polysorbates 20, 60, 65, and 80

Several reports have described the applications of qNMR to determine specific types of chemical compound, such as natural products, impurities, and polymers (Stefanova et al. 1988; Paula 2001; Jake et al. 2002; Wells et al. 2002; Paula et al. 2005). Recently, a practical set of parameters for qNMR has been discussed (Saito et al. 2004). Furthermore, qNMR using an internal standard has been suggested as a new way of determining the contents of surfactants with a relatively high throughput (Koike et al. 2004a, 2004b, 2005). To minimize quantitative errors, we used the qNMR conditions described by Koike and colleagues, as listed in Table I. In particular, the flip angle was set to 45°, and the spectral width was set at a value sufficient for the peak of interest to fall within 80% of its centre, because the signal intensities decreased towards both edges of the spectral window. The number of data points was set at 64 000 to enhance the resolution. The pulse delay was set at up to 30 s, as high-precision NMR can only be achieved when the pulse delay time is greater than the quintuple spin-lattice relaxation time ($>5 * T_1$). As qNMR is based on the fact that the signal intensities of a given resonance are directly proportional to the molar quantity of the nucleus within the sample, the EO signal intensity of polysorbates and four protons on the benzene ring of PHP were used to determine the EO contents. The total time taken to obtain one FID using these parameters was <10 min.

The weight percentage of the EO groups was calculated according to Equation 1.

$$\mathrm{EO(w/w\%)} = \frac{(I_{EO}/H_{EO} \times M_{EO}/W_{sample})}{(I_{standard}/H_{standard} \times M_{standard}/W_{standard})} \times 100. \quad (1)$$

Here, I_{EO} is the signal intensity of the EO group; H_{EO} is the number of protons of the EO group (four); M_{EO} is the partial molecular weight of the EO group (44); W_{sample} is the weight (mg) of the sample in 3 ml of NMR solvent including PHP as an internal standard; $I_{standard}$ is the total signal intensity of PHP; $H_{standard}$ is the number of protons on the benzene ring of PHP (four); $M_{standard}$ is the molecular weight of PHP (204); and $W_{standard}$ is the weight (mg) of PHP in 3 ml of NMR solvent.

We initially confirmed that the qNMR showed linearity between the intensity of the EO signal and the amount of polysorbate 20. Various amounts of the reagent-grade polysorbate 20 sample were analysed by ^{1}H-NMR under the conditions described in the Materials and methods and Table I. The NMR spectrum of polysorbate 20 with the internal standard is shown in Figure 4. The four protons of the PHP benzene ring were observed as two double–doublet signals at δ_H values of 7.46–7.66 ppm and 8.18–8.38 ppm, respectively. The ratio of the EO signal intensity was calculated as follows: intensity of EO/total intensities of four protons on PHP benzene ring. The relationship between EO/PHP and the amount of polysorbate 20 was linear ($R^2 = 0.9996$) in the range of 12.5–100 mg of polysorbate 20 in 3 ml of NMR solvent. Based on these results, we concluded

Table II. Determination of EO contents in polysorbates by qNMR.[a]

Sample name	MERCURY (400 MHz, VARIAN)			ECA500 (500 MHz, JEOL)		
	Entry	EO (%)	SD	Entry	EO (%)	SD
Polysorbate 20 (polyoxyethylene (20) sorbitan monolaurate)	1	73.0		1	72.2	
	2	71.8		2	71.8	
	3	73.2		3	72.3	
	4	71.7		4	72.5	
	5	71.9		5	71.6	
				6	72.9	
				7	72.0	
				8	72.7	
				9	73.7	
	AV	72.3	0.7	AV	72.4	0.6
Polysorbate 60 (polyoxyethylene (20) sorbitan monostearate)	1	67.7		1	67.4	
	2	65.3		2	67.7	
	3	68.9		3	67.5	
	4	67.8		4	67.9	
	5	66.9		5	68.6	
	AV	67.3	1.3	AV	67.8	0.5
Polysorbate 65 (polyoxyethylene(20) sorbitan tristearate)	1	49.1		1	49.8	
	2	49.8				
	3	49.5				
	4	49.8				
	5	48.7				
	AV	49.4	0.5			
Polysorbate 80 (polyoxyethylene (20) sorbitan monooleate)	1	65.0		1	67.0	
	2	65.5				
	3	66.2				
	4	64.8				
	5	65.1				
	AV	65.3	0.6			

[a]Reagent-grade polysorbates were purchased from Wako Pure Chemical Industries, Ltd. "Entry" means that the same sample was measured repeatedly on different days.

that qNMR could quantitatively determine the EO contents of polysorbates.

In order to verify whether qNMR could accurately determine the EO contents of polysorbates, two different NMR instruments (MERCURY and ECA500, with magnetic field strengths of 400 and 500 MHz, respectively) were used to repeatedly measure the EO contents of reagent grade polysorbates 20, 60, 65, and 80, which are generally used as standards. The results are shown in Table II. Reproducible results were obtained from each sample using the MERCURY system. Furthermore, the results obtained by the two NMR instruments did not differ significantly (standard deviations = 0.5–1.3%). These findings confirmed that it was possible to determine the EO contents of polysorbates using this approach regardless of the NMR instrument employed.

Finally, to confirm the validity of qNMR, we determined the EO contents of the commercially synthesized polysorbates 20, 60, 65, and 80, which met the specifications of the JECFA. All of the EO contents of the polysorbates were within the limits described in the *Compendium of Food Additive and Flavoring Agent Specifications* (JECFA [internet]) (Table III). The qNMR method for determining the EO contents of polysorbates demonstrated in this paper thus represents a simple and rapid alternative to the classic titration method recommended by the JECFA, which does not require specific chemical reactions or sophisticated apparatus. Moreover, the qNMR method made it possible to distinguish between Polysorbates 60 and 80, which have the same stipulated value, by comparison with the ^{1}H-NMR spectra, as polysorbate 80 consisting of an unsaturated fatty acid only showed the signals of olefinic protons at δ_H 5.3 ppm. It is theoretically possible to determine the ratio of a substituted group in any molecule, or the quality of any compound, using the proposed qNMR method with an internal standard, provided that the target proton signals can be separated from those

Table III. EO contents in commercial polysorbates determined using qNMR.[a]

Name	Stipulated value	Brand	EO (%)	SD
Polysorbate 20 (polyoxyethylene (20) sorbitan monolaurate)	70.0–74.0%	A	71.2	
		B	73.0	
		C	70.3	
		D	71.0	
		E	71.5	
		AV	71.4	1.0
Polysorbate 60 (polyoxyethylene (20) sorbitan monostearate)	65.0–69.5%	A	66.9	
		B	65.4	
		C	68.0	
		D	68.1	
		E	67.2	
		AV	67.1	1.1
Polysorbate 65 (polyoxyethylene (20) sorbitan tristearate)	46.0–50.0%	A	48.3	
		B	46.0	
		C	–	
		D	47.2	
		E	48.1	
		AV	47.4	1.1
Polysorbate 80 (polyoxyethylene (20) sorbitan monooleate)	65.0–69.5%	A	67.4	
		B	65.1	
		C	69.3	
		D	66.7	
		E	68.0	
		AV	67.1	1.6

[a]Brands A–E were purchased from five manufacturers. Brand C does not supply polysorbate 65.

of non-target groups and impurities. We are currently investigating the potential for this technique to determine various other compounds and polymers.

Conclusions

This research demonstrated that the EO contents of commercial polysorbates 20, 60, 65, and 80 could be readily determined using qNMR with an internal standard. Clear NMR data for the polysorbates were obtained from simple sample preparations. Two different NMR instruments validated the proposed method, and no significant differences were observed among the results. Moreover, the data obtained for commercial polysorbates 20, 60, 65, and 80 were in good agreement with the JECFA guidelines.

It is generally difficult to determine the amounts of substituted groups within polymers owing to their great diversity in molecular weights and structures. Classical methods require time-consuming preparation to set up the apparatus, and technically skilled operators. Furthermore, as there are no alternative methods to validate the results, they have to be accepted without verification. Our proposed qNMR is a rapid and simple analysis that provides the structural information of target compounds together. These advantages will reduce dramatically the time and manpower cost required, even if the NMR spectrometer and the solvents are expensive. qNMR is thus a valuable additional and/or alternative method, with a broad range of applications in quantitative analysis.

Acknowledgements

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