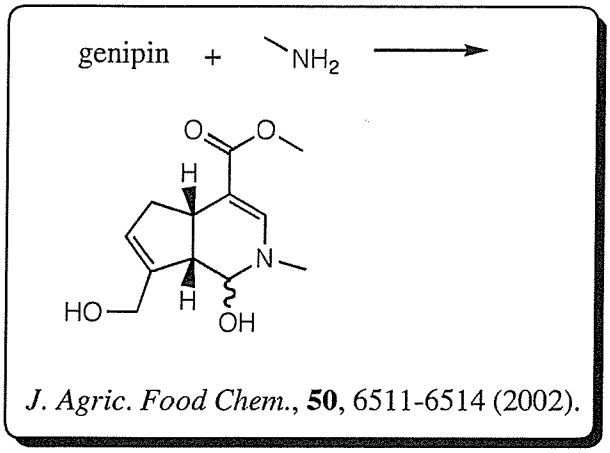
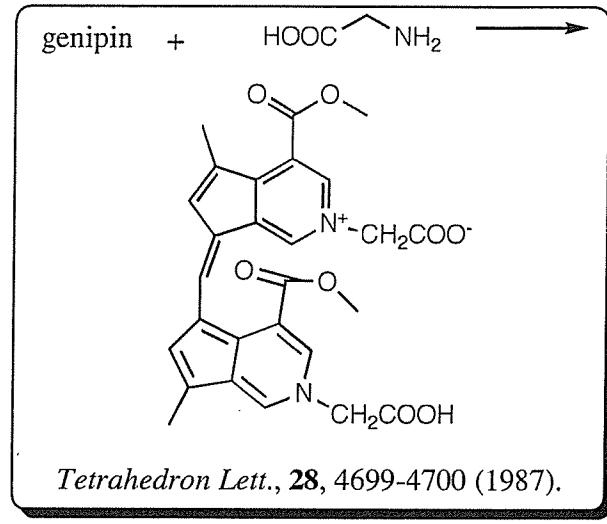
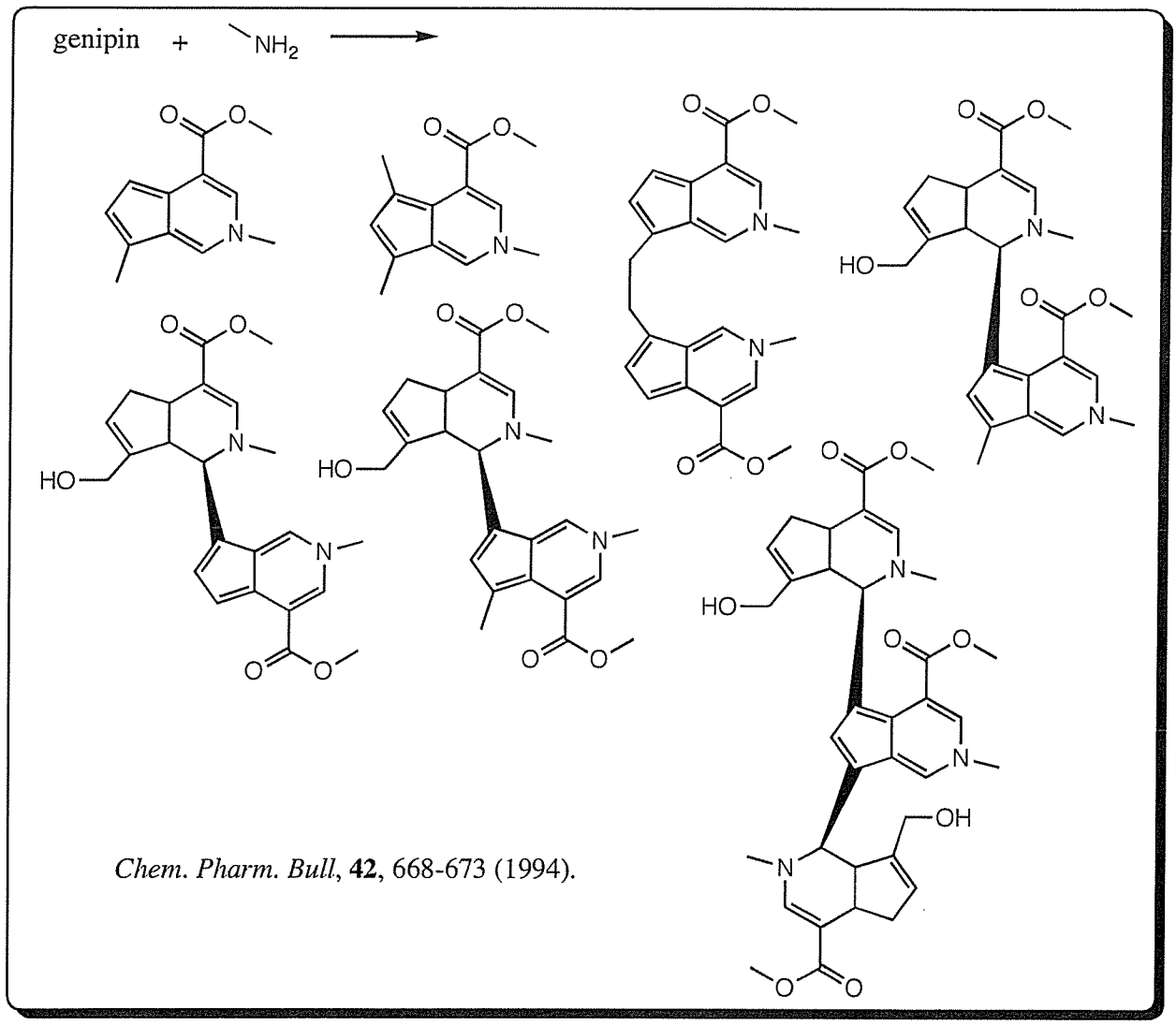


J. Agric. Food Chem., **50**, 6511-6514 (2002).



Tetrahedron Lett., **28**, 4699-4700 (1987).



Chem. Pharm. Bull, **42**, 668-673 (1994).

Figure G4. Reported intermediates of Gardenia blue

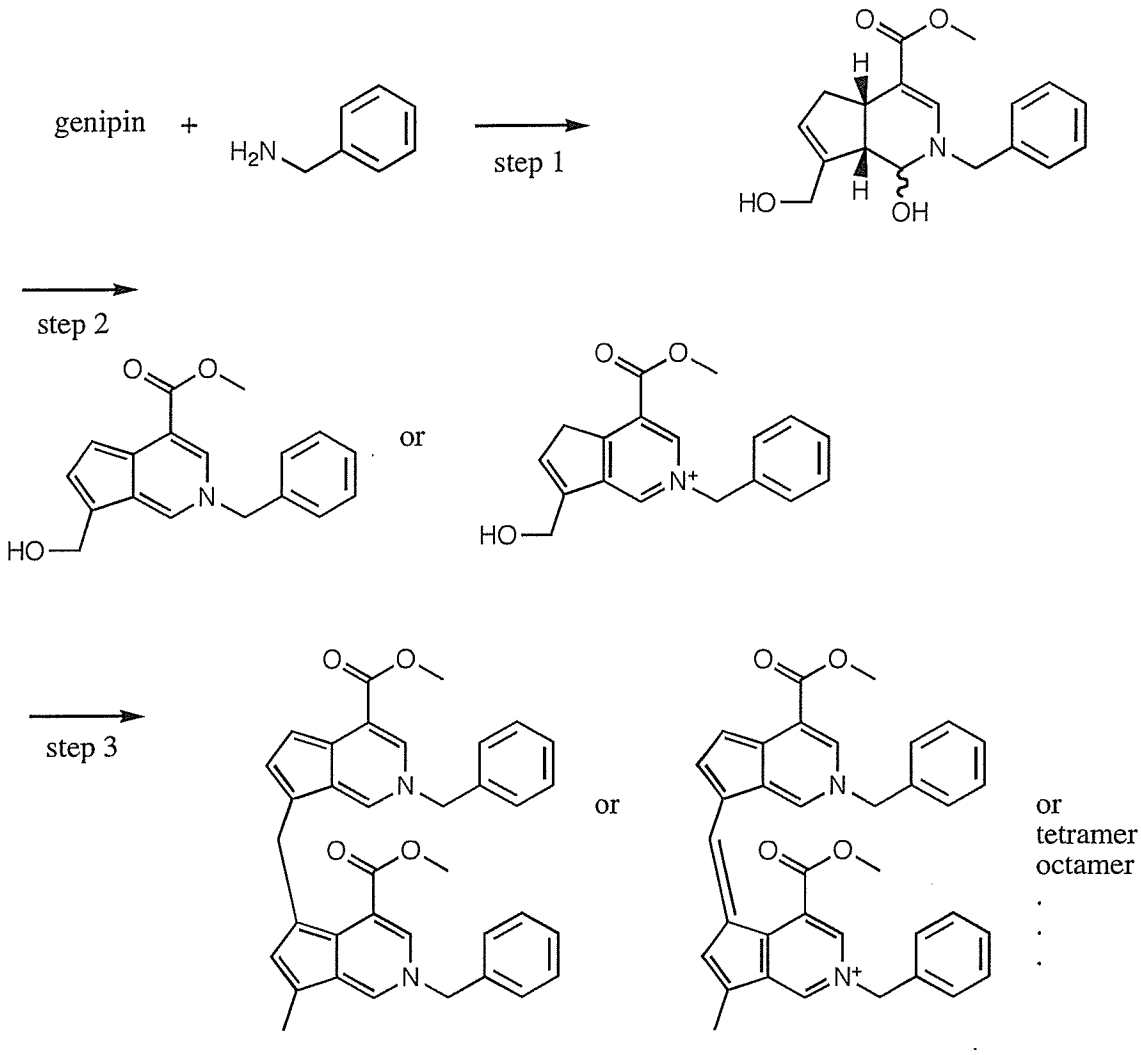
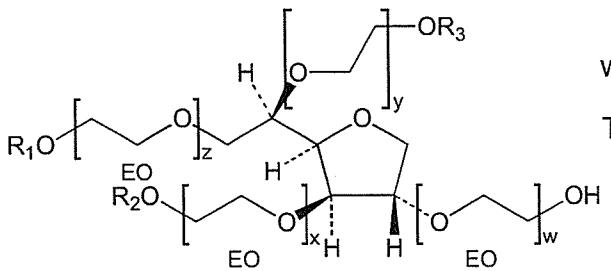


Figure G5. Proposed structures of main compounds



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)

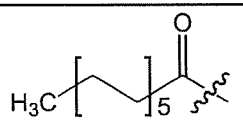
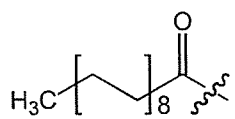
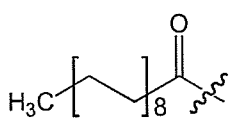
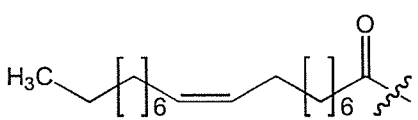
Compound			Formula (MW)	EO(%) in molecule
Polysorbate 20 (polyoxyethylene (20) sorbitan monolaurate)	R_1= 	$R_2 = R_3 = H$	$C_{58}H_{114}O_{26}$ (MW1227.5)	EO(%) = 71.8
Polysorbate 60 (polyoxyethylene (20) sorbitan monostearate)	R_1= 	$R_2 = R_3 = H$	$C_{64}H_{126}O_{26}$ (MW1311.7)	EO(%) = 67.2
Polysorbate 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 E1. 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.

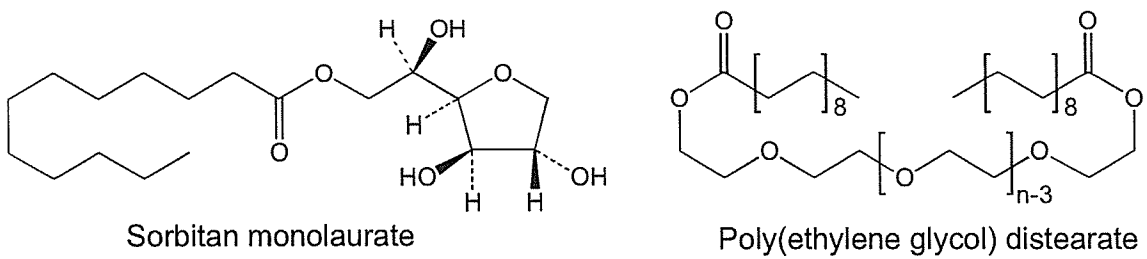


Figure E2. Structures of sorbitan monolaurate and poly(ethylene glycol) distearate.

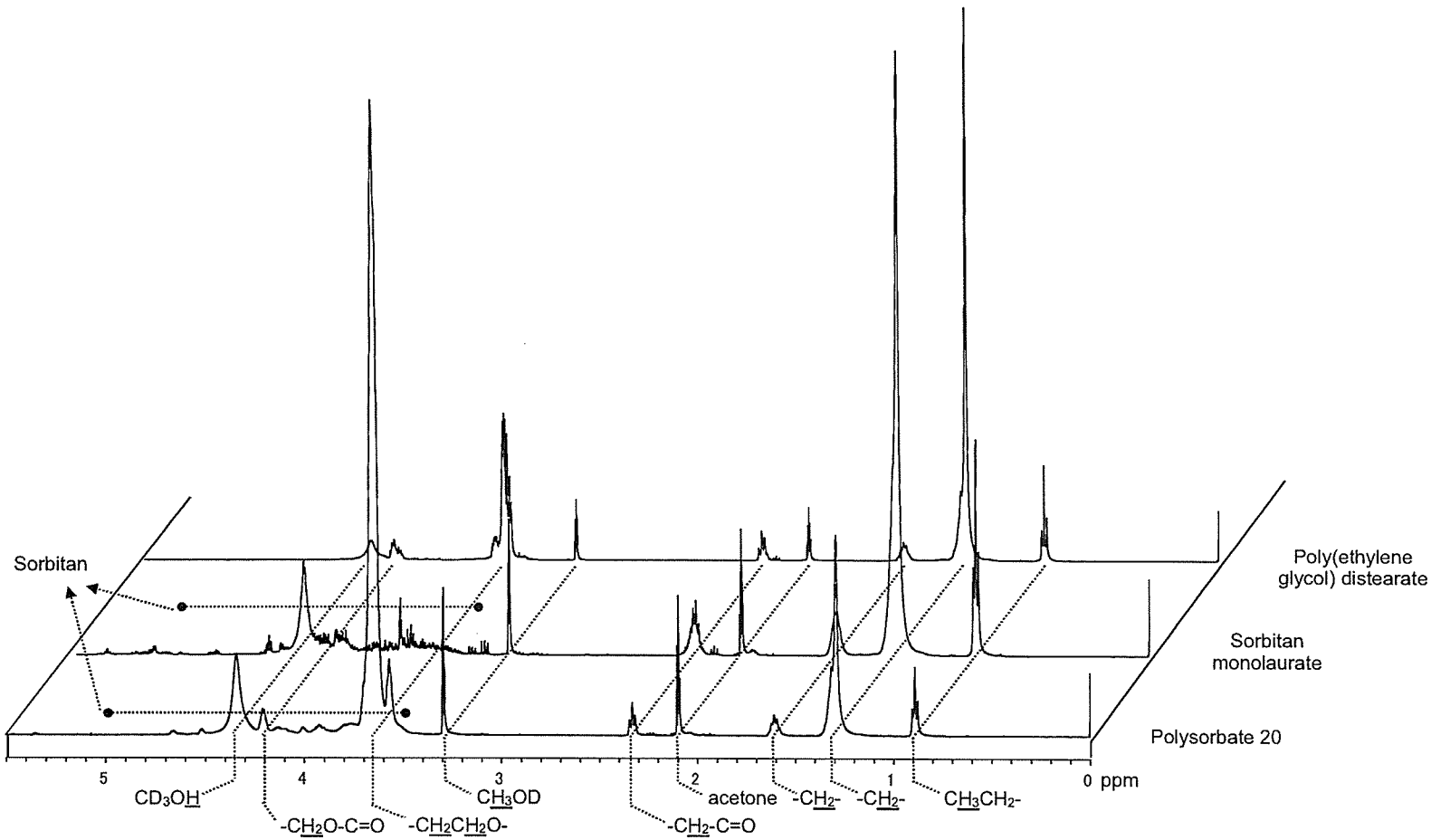


Figure E3. Comparison of NMR spectra of polysorbate 20, sorbitan monolaurate and poly(ethylene glycol) distearate. ^{1}H-NMR spectra were obtained using the ECA500 system (500 MHz; JEOL) under the conditions shown in Table I.

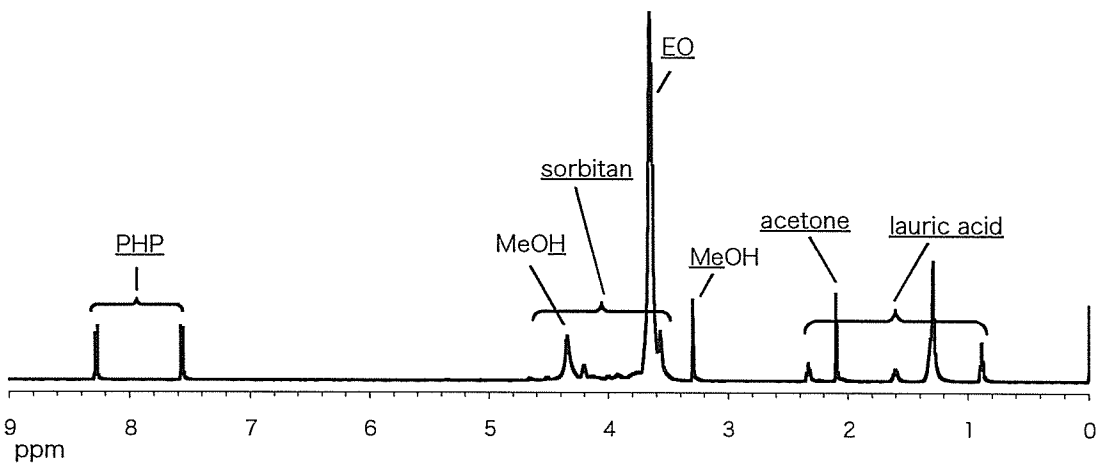


Figure E4. ^{1}H-NMR spectrum of polysorbate 20.
The spectrum was obtained using the ECA500 system (500 MHz; JEOL). PHP was added as an internal standard. Signals of the four protons on the benzene ring of PHP were observed at δ_H values of 7.46-7.66 ppm and 8.18-8.38 ppm. Most of the EO signals of polysorbate 20 were observed in a large envelope between δ_H 3.40 and 3.85 ppm.

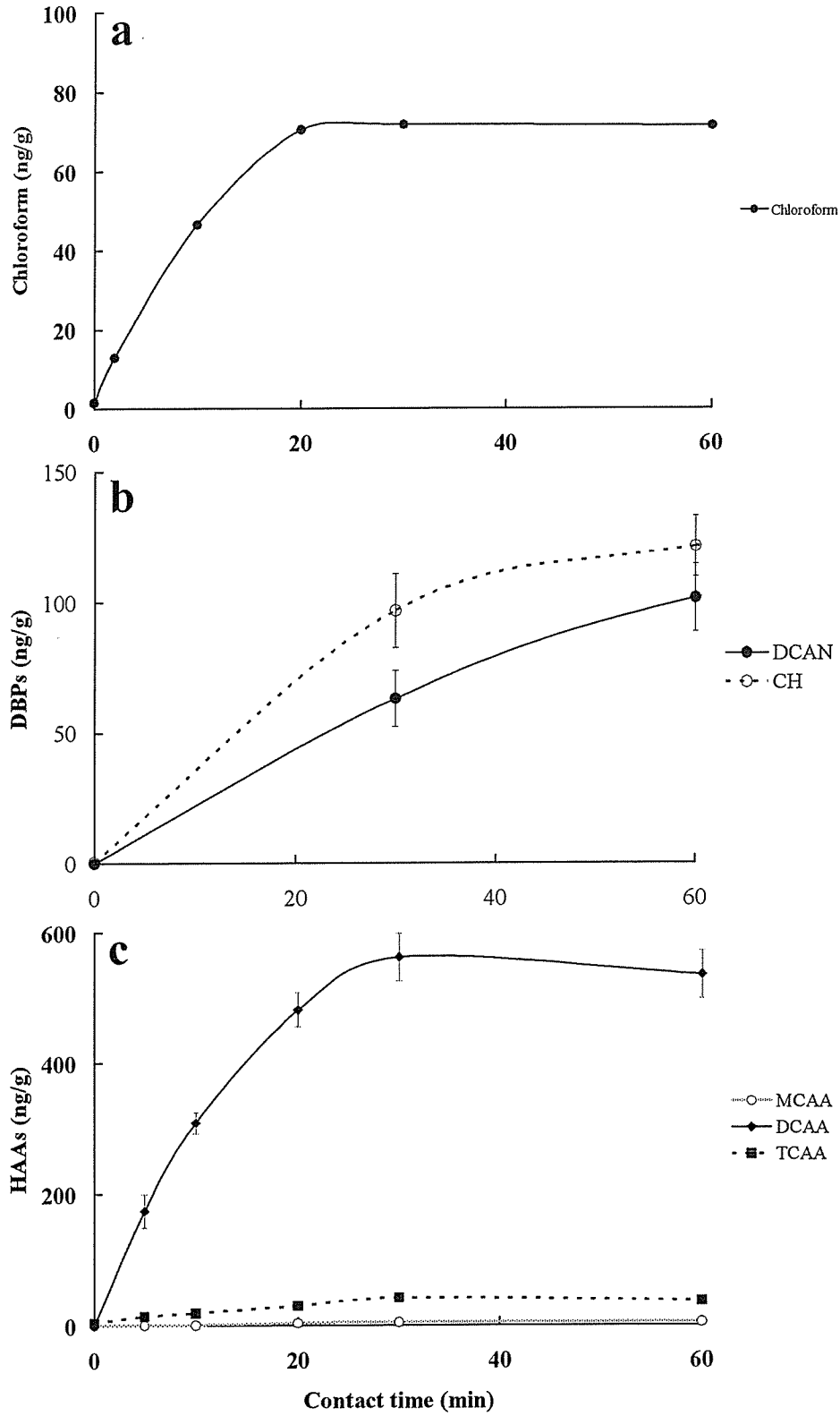


Fig.1. 次亜塩素酸ナトリウム処理カット野菜中における消毒副生成物生成量の経時変化. a)クロロホルム, b)ハロアセトニトリル及び抱水クロラール, c)ハロ酢酸

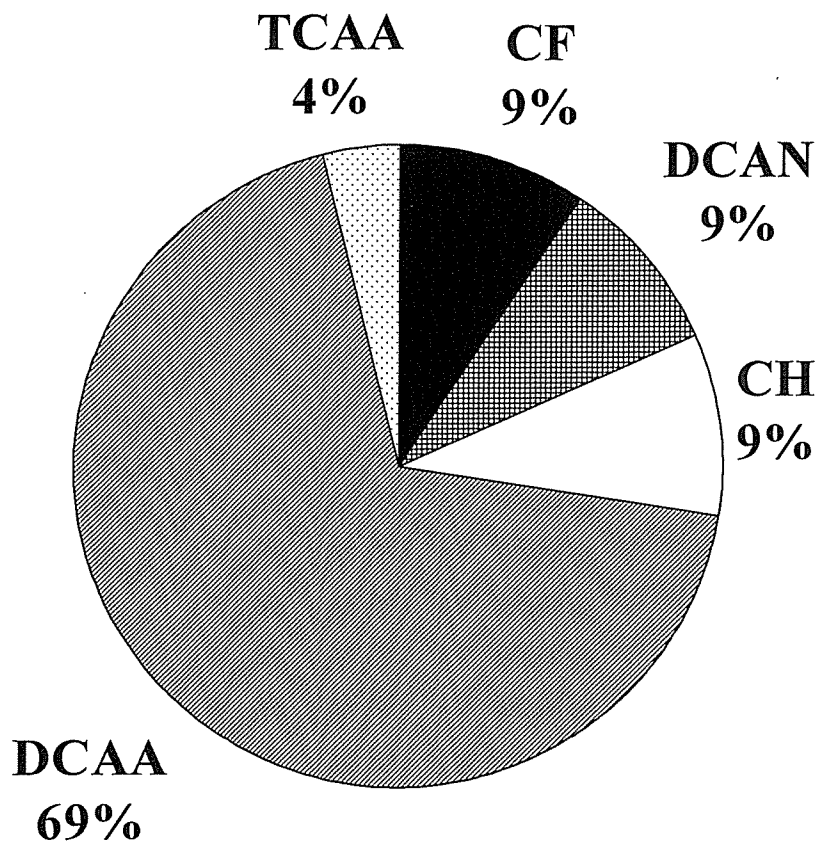


Fig.2. 次亜塩素酸ナトリウム処理カット野菜中における消毒副生成物のモル構成比

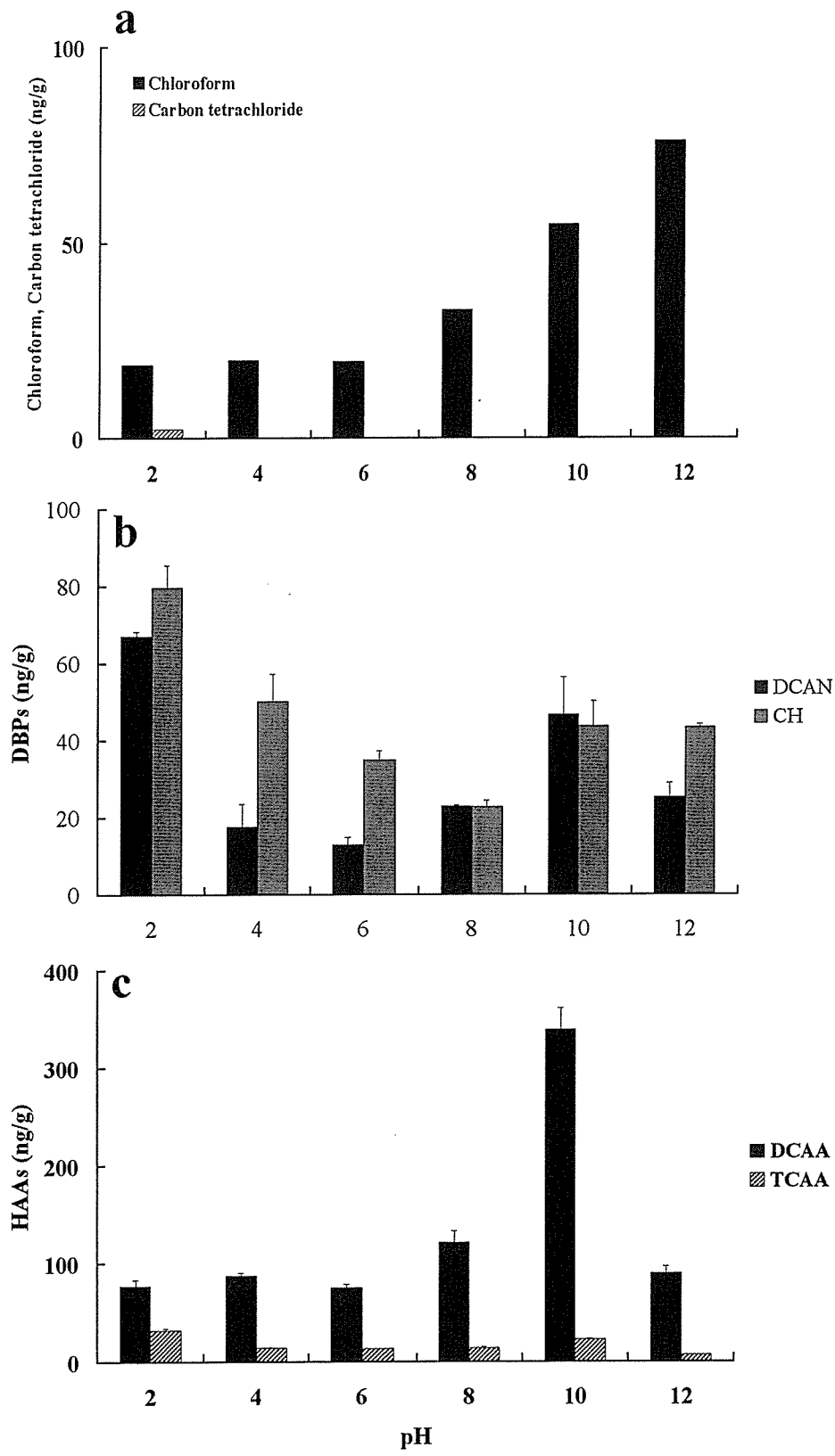


Fig.3. 次亜塩素酸ナトリウム浸漬液の pH によるカット野菜中のハロ酢酸生成量の変化. a)クロロホルム, b)ハロアセトニトリル及び抱水クロラール, c)ハロ酢酸

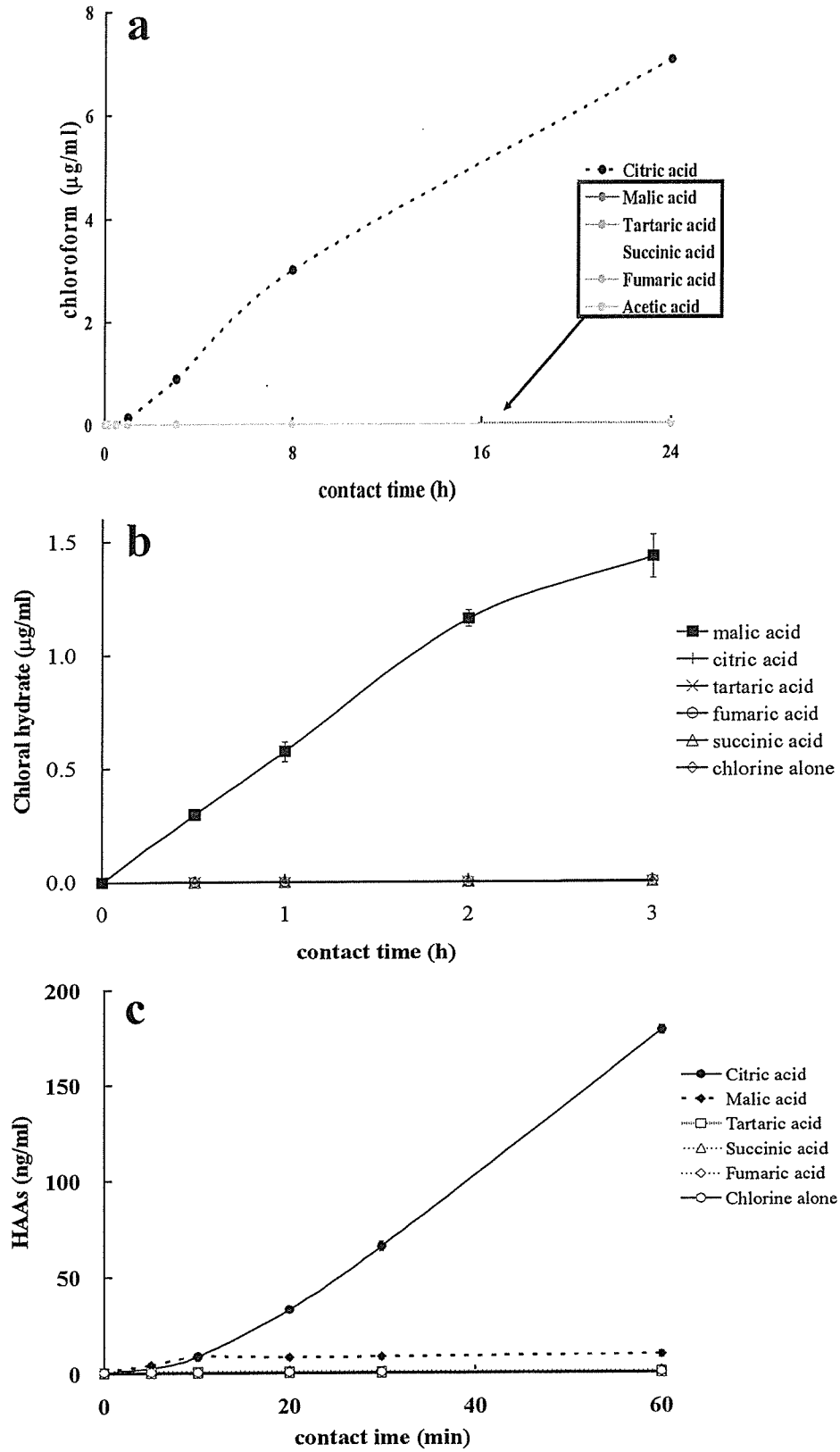


Fig.4. 次亜塩素酸ナトリウム・有機酸混合溶液における有機酸混和後の消毒副生成物の推移.a)クロロホルム, b)抱水クロラール, c)ハロ酢酸

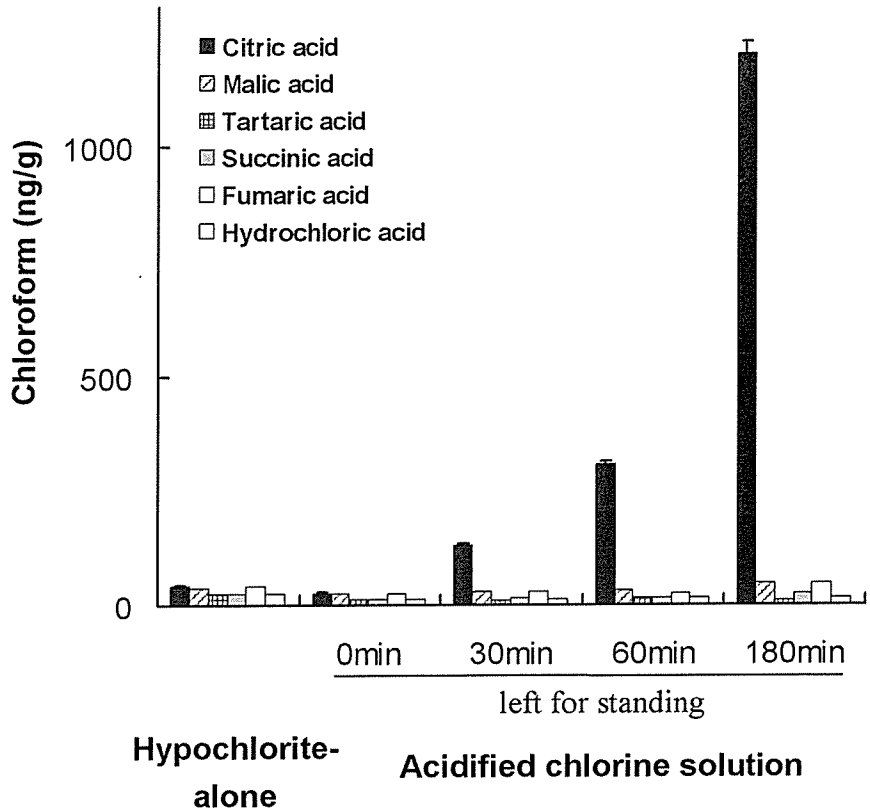
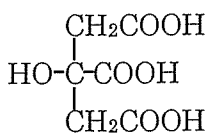
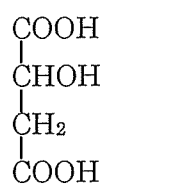


Fig.5. 各種酸共存下における次亜塩素酸処理カットキャベツ中のクロロホルム生成量

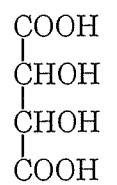
クエン酸



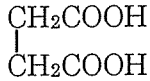
DL-リンゴ酸



DL-酒石酸



コハク酸



フマル酸

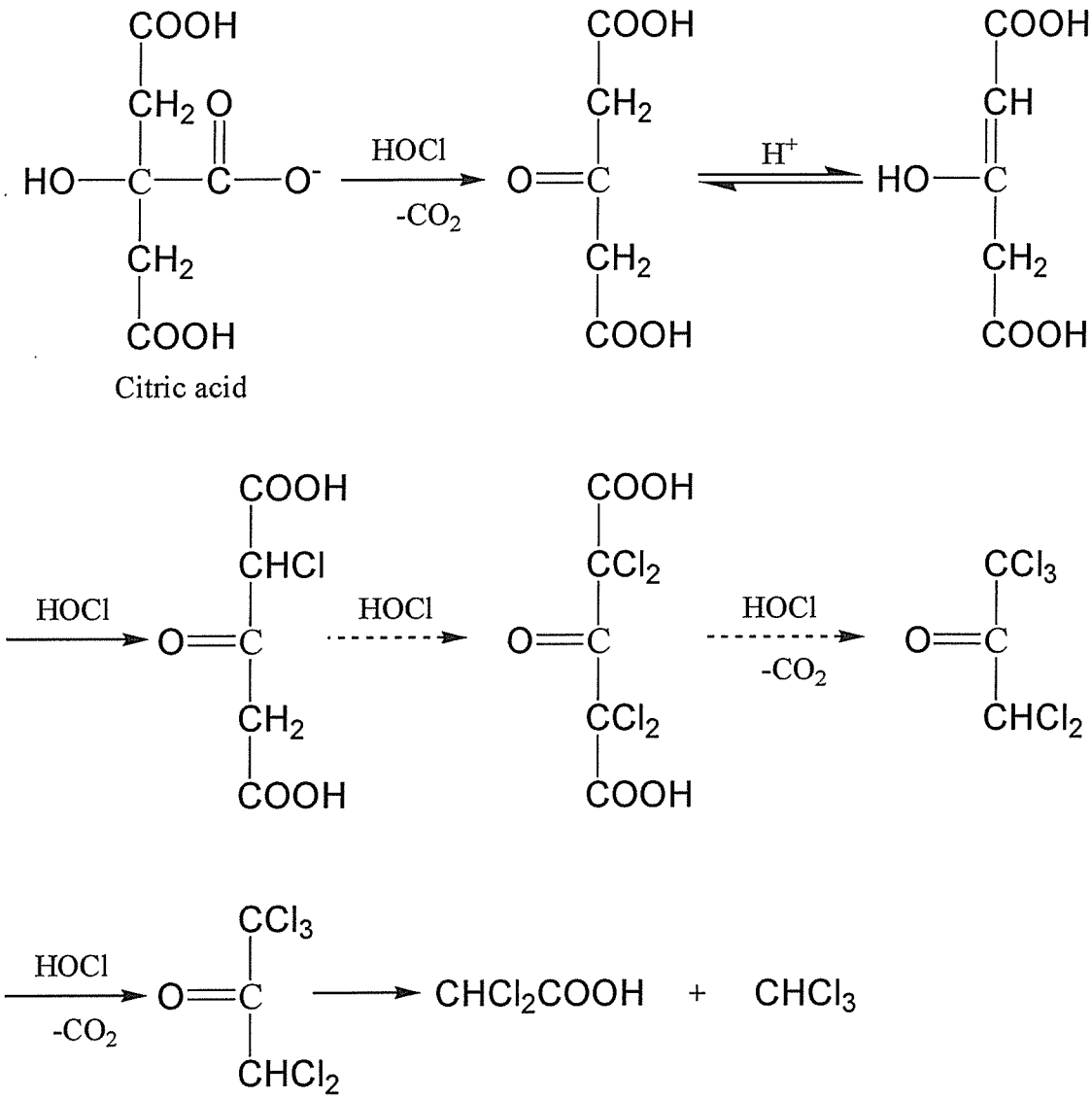


Fig.6. クエン酸の次亜塩素酸ナトリウムによるクロロホルム及びジクロロ酢酸生成メカニズム(仮説)

Table 1 新規指定品の摂取量

品目名	使用量 [kg]	推定摂取量※ [μg/人/日]		
		日本	米国	欧州
isopropyl alcohol	173.3	44	10968	85510
isobutanol	24.0	6	290	530
2,3,5,6-tetramethylpyrazine	16.7	4	19	8
2,3,5-trimethylpyrazine	14.9	4	46	120
propyl alcohol	12.7	3	549	360
isoamyl alcohol	5.6	1	2194	1581
amyl alcohol	1.3	0.3	34	83
3,5(6)-dimethyl-2-ethylpyrazine	0.6	0.2	9	44

※欧米の摂取量は食品安全委員会の審議時に使用した値
(http://www.fsc.go.jp/hyouka/hy_tenkabutu.html)

Table P1. NMR data (δ, ppm, D_2O:CH_3CN=20:80) of peak A and B.

No.	Peak A 2-ethyl-3,5-dimethylpyrazine (**1**)			Peak B 2-ethyl-3,6-dimethylpyrazine (**2**)		
	^1H	^{13}C	HMBC	^1H	^{13}C	HMBC
2	-	161.7		-	162.0	
3	-	155.0		-	155.0	
5	-	155.0		8.36 (1H, s)	141.9	C-3, C-6
6	8.38 (1H, s)	141.3	C-2	-	154.5	
3-$\underline{CH_3}$-	2.68 (3H, s)	20.9	C-2, C-3	2.69 (3H, s)	20.9	C-2, C-3
6-$\underline{CH_3}$-	-	-		2.63 (3H, s)	20.9	C-5, C-6
5-$\underline{CH_3}$-	2.62 (3H, s)	20.7	C-5, C-6	-	-	
2-$\underline{CH_3}$-CH_2-	1.38 (3H, t, J = 7.5 Hz)	12.7	C-2	1.37 (3H, t, J = 7.5 Hz)	13.1	C-2
2-CH_3-$\underline{CH_2}$-	2.98 (2H, q, J = 7.5 Hz)	27.8	C-2	2.97 (2H, q, J = 7.5 Hz)	27.9	C-2

a) ^{13}C chemical shifts were assigned indirectly by HMQC and HMBC.

Table P2. Composition rate of 2-ethyl-3,5-dimethylpyrazine (**1**) and 2-ethyl-3,6-dimethylpyrazine (**2**) derived from GC-MS, LC-PDA-MS and NMR

	detection	**1** (%)	**2** (%)
GC/MS	TIC	56.6	43.4
PDA-LC/MS	UV 278 nm	55.0	45.0
NMR	δ 8.38 and 8.36 ppm	56.5	43.5

Table E1. 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	64000
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 phhtalate (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

Table E2. Determination of EO contents in polysorbates by qNMR.

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	0.4
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			

Reagent grade polysorbates were purchase from Wako Pure Chemical Industries, Ltd. The "entry" means the same sample was measured repeatedly on different days.

Table E3. EO contents in commercial polysorbates determined using qNMR.

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

Brands A~E were purchased from five manufacturers.

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., Yomota, C., Furusho, N., Sato, K., Yamazaki, T., Tanamoto, K.,	Application of liquid chromatography-nuclear magnetic resonance *spectroscopy* to the identification of ethyldimethylpyrazine, a food flavoring agent	Food Add. Contam.	23(12)	1253-1259	2006
Kitamura, Y., Iwasaki T., Saito, M., Mifune, M., Saito, Y., Sato, K., Yomota C., Tanamoto K	Standard Infrared Absorption Spectrum of Betaine and Optimal Conditions for its Measurement	J. Food Hygienics Society of Japan	47(5)	232-236	2006
Sugimoto, N., Koike, R., Furusho, N., Tanno, M., Yomota, C., Sato, K., Yamazaki, T., Tanamoto, K.,	Quantitative nuclear magnetic resonance spectroscopy determination of the oxyethylene group contents of polysorbates	Food Add. Contam.	accepted		2007
Mine, T., Okada, Y. and Semma, M	The interaction of sorbic acid with amino acid may alter the quality of foods somewhere in the food chain from production to table	Jpn. J.Food Chem.	In press		2007

Food Additives and Contaminants, December 2006; 23(12): 1253–1259

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Application of liquid chromatography–nuclear magnetic resonance spectroscopy for the identification of ethyldimethylpyrazine, a food flavouring agent

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(Received 24 April 2006; revised 20 June 2006; accepted 20 June 2006)

Abstract
The application of liquid chromatography–nuclear magnetic resonance spectroscopy (LC–NMR) for the direct identification of ethyldimethylpyrazine, a food flavouring agent, has been studied. The commercial product is a mixture of two regio-isomers, 2-ethyl-3,5-dimethylpyrazine (1) and 2-ethyl-3,6-dimethylpyrazine (2); however, the exact composition of the mixture is unknown. Structural characterization by LC–MS and GC–MS was not possible because both regio-isomers yield the same molecular related ion and ion fragmentation. To rapidly identify the two regio-isomers, the product was analyzed by LC–NMR with on-flow and fraction loop modes. From the results, the structure elucidations of the two regio-isomers could be carried out without the need to isolate the isomers by the usual procedures.

Keywords: *Food additive, dimethylethylpyrazine, flavouring agent, LC–MS, GC–MS, LC–NMR*

Introduction

Recently, gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–mass spectrometry (LC–MS) have been widely exploited, with a massive growth of applications, particularly in food safety, and especially in the identification and quantification of bioactive constituents, food additives and contaminants. Despite these advances, MS by itself does not always provide definitive structural identification, and nuclear magnetic resonance (NMR) spectroscopic data are often required. NMR has played an important role in analytical chemistry but conventional NMR spectroscopic analysis has required time-consuming isolation and purification steps. Recently, on-line liquid chromatography–nuclear magnetic resonance (LC–NMR) has been developed and can provide structural information that complements LC–MS and GC–MS data, facilitating rapid analyses of mixtures without the need for isolation. The applications of LC–NMR to drug metabolism, natural products identification and characterization of isomeric mixtures have been reviewed (Wolfender et al. 1998, 2001; Iwasa et al. 2003; Sohda et al. 2004; Waridel et al. 2004).

Ethyldimethylpyrazine is a flavouring agent permitted for use in foods since December 2004 in Japan. The substance is used widely for adding the flavour of roasted nuts to chocolate, cookies, etc. (Goldman et al. 1967; Ohta et al. 1987; Buchi and Galindo 1991; Burdock 1997). The commercial flavouring agent product consists of two regio-isomers, 2-ethyl-3,5-dimethylpyrazine (1) and 2-ethyl-3,6-dimethylpyrazine (2), as shown in Figure 1. FAO/WHO Joint Expert Committee on Food Additives (JECFA) had evaluated a mixture of two isomers of ethyldimethylpyrazine. JECFA concluded this flavouring agent was of "no safety concern" based on current intake, but safety evaluation of each isomer was not carried out (WHO Food Additives Series 48). The relative amounts of 1 and 2 in the commercial product are not specified, and the composition of the mixture is

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