用途名	食添NO.	食品添加物名	章	純食品向け出荷量（Kg）	純食品向け査定量（Kg）	摂取量（Kg）	1人一日摂取量(mg/人/日)	ADI 1)(mg/人/日)	ADI比(A/B %)	注
無機化合物（その他）	193	炭酸マグネシウム	22	699,620	699,620	559,696	12.00			
防ばい剤	194	チアベンダゾール	9	0	0	0	0			
強化剤（ビタミンB1）	195	チアミン塩酸塩	14	44,000	16,000	7,680	0.164			
強化剤（ビタミンB1）	196	チアミン硝酸塩	14	63,300	20,000	10,185	0.218			
強化剤（ビタミンB1）	197	チアミンセチル硫酸塩	14	0	0	0	0			
強化剤（ビタミンB1）	198	チアミンチオシアン酸塩	14	0	0	0	0			
強化剤（ビタミンB1）	199	チアミンナフタレン-1,5-ジスルホン酸塩	14	0	0	0	0			
強化剤（ビタミンB1）	200	チアミンラウリル硫酸塩	14	10,000	10,000	2,460	0.053			
香料	201	チオエーテル類	15	16,543	16,000	12,800	0.270			
香料	202	チオール類	15	213	200	160	0.0034			
調味料	203	L-テアニン	11	22,000	22,000	17,600	0.376			
香料	204	デカナール	15	604	600	480	0.0100			
香料	205	デカノール	15	106	100	80	0.0017			
香料	206	デカン酸エチル	15	971	1,000	800	0.0140			
着色料	207	鉄クロロフィリンナトリウム	3	130	130	104	0.0022	設定なし		
保存料	209	デヒドロ酢酸ナトリウム	4	50,000	50,000	40,000	[0.757]		〃	22)
香料	210	テルピネオール	15	1,834	2,500	2,000	0.0430			
香料	211	テルペン系炭化水素類	15	45,959	46,000	36,800	0.790			
糊料	212	デンプングリコール酸ナトリウム	6	300	300	240	0.01			
糊料	213	デンプンリン酸エステルナトリウム	6	0	0	0	0			
有機酸類	214-1	グルコン酸銅	17	2,000	1,700	200				11)
無機化合物（その他）	214-2	硫酸銅	22	270	270	216	0.004			
着色料	215	銅クロロフィリンナトリウム	3	4,700	5,000	4,000	0.086	750	0.012	
着色料	216	銅クロロフィル	3	7,140	7,140	5,712	0.122	750	0.016	
酸化防止剤	217	dl-α-トコフェロール	7	16,300	16,300	13,000	0.028	100	0.03	
強化剤（アミノ酸系）	218	DL-トリプトファン	13	0	0	0	0			
強化剤（アミノ酸系）	219	L-トリプトファン	13	2,100	2,100	1,700	0.036			
強化剤（アミノ酸系）	221	DL-トレオニン	13	2,000	2,000	1,600	0.034			
強化剤（アミノ酸系）	222	L-トレオニン	13	6,500	6,500	5,200	0.110			
その他用途添加物	223	ナトリウムメトキシド	16	0	0	0	0			
強化剤（ナイアシン）	224	ニコチン酸	14	4,900	4,900	2,940	0.063			

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強化剤（ナイアシン）	225	ニコチン酸アミド	14	130,400	130,400	63,218	1.353			
漂白剤	226	二酸化硫黄	5	532,000	60,000	48,000	☆1.03	二酸化硫黄として 35	19.9	23)
その他用途添加物（小麦粉改良剤）	227	二酸化塩素	16	0	0	0	0			
無機化合物（その他）	228	二酸化ケイ素	22	1,383,620	−	0			−	
無機化合物（その他）	228-1	微粒二酸化ケイ素		136,690	31,000	24,800	0.530	特定せず		
無機化合物（その他）	229	二酸化炭素	22	147,491,000	147,491,000	13,181,100	288.3	特定せず	−	
着色料	230	二酸化チタン	3	6,800	4,350	3,600	0.074	制限せず		
有機酸類（酸味料）	231	乳酸	17	7,030,000	4,000,000	3,200,000	68.5			24)
有機酸類（強化剤）	232	乳酸カルシウム	17	2,800,000	2,800,000	2,240,000	47.9			25)
有機酸類（強化剤）	233	乳酸鉄	17	400	400	300	0			25)
有機酸類（調味料）	234	乳酸ナトリウム（60%）	17	2,300,000	2,300,000	1,104,000	29.5			25,26)
香料	235	γ-ノナラクトン	15	2,507	2,500	2,000	0.0430	62.5	0.067	
着色料	236	ノルビキシンカリウム	3	51,701	11,285	9,028	0.190	30	0.64	27)
着色料	237	ノルビキシンナトリウム	3	150	−	−	−	30		27)
香料	238	バニリン	15	51,578	110,000	88,000	1.884			
保存料	239	パラオキシ安息香酸イソプチル	4	3,500	3,500	2,800	〔0.175〕		〔0.04〕	28)
保存料	240	パラオキシ安息香酸イソプロピル	4	4,500	4,500	3,600				28)
保存料	241	パラオキシ安息香酸エチル	4	0	0	0		500※		28)
保存料	242	パラオキシ安息香酸ブチル	4	6,000	6,000	4,800		※エチル，メチル及びプロピルエステルのGroup ADI		28)
保存料	243	パラオキシ安息香酸プロピル	4	0	0	0				28)
香料	244	パラメチルアセトフェノン	15	47	50	40	0.0009			
強化剤（アミノ酸系）	245	L-バリン	13	106,400	106,400	85,100	1.820			
強化剤（パントテン酸）	246	パントテン酸カルシウム	14	38,000	38,000	27,885	0.447			
強化剤（パントテン酸）	247	パントテン酸ナトリウム	14	0	0	0	0			
強化剤（アミノ酸系）	249	L-ヒスチジン塩酸塩	13	15,600	15,600	12,500	0.27			
強化剤（ビタミンB1）	250	ビスベンチアミン	14	0	0	0	0			
強化剤（ビタミンA）	251	ビタミンA	14	0	0	0	0			29,30)
強化剤（ビタミンA）	252	ビタミンA脂肪酸エステル	14	15,900	15,900	7,632	0.163			
香料	253	ヒドロキシシシトロネラール	15	290	300	240	0.0051			
香料	254	ヒドロキシシシトロネラールジメチルアセタール	15	27	30	24	0.0005			
香料	257	ピペロナール	15	1,063	626	568	0.0110			

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その他用途添加物（防虫剤）	258	ピペロニルブトキシド	16	0	0	0	0			
有機酸類（酸味料）	259	氷酢酸	17	1,200,000	5,700,000	4,560,000	97.6			
強化剤	260	ピリドキシン塩酸塩	14	113,900	21,400	10,567	0.226			31)
漂白剤	261	ピロ亜硫酸カリウム	5	0	15,000	12,000	☆0.26			32)
漂白剤	262	ピロ亜硫酸ナトリウム	5	228,000	230,000	184,000	☆3.94			32)
無機化合物（リン酸化合物）	263	ピロリン酸四カリウム	19	701,000	150,000	120,000	2.57			33)
無機化合物（カルシウム剤）	264	ピロリン酸二水素カルシウム	18	87,000	90,000	72,000	1.54	3500	0.04	33)
無機化合物（リン酸化合物）	265	ピロリン酸二水素二ナトリウム	19	1,371,000	700,000	560,000	12.0			33)
無機化合物（その他）	266	ピロリン酸第二鉄	22	39,900	39,900	31,92	0.68			33)
無機化合物（リン酸化合物）	267	ピロリン酸四ナトリウム（無水）	19	2,069,000	1,000,000	800,000	17.12			33)
強化剤（アミノ酸系）	268	L-フェニルアラニン	13	5,000	5,000	4,000	0.086			
香料	269	フェニル酢酸イソアミル	15	71	70	56	0.0012			
香料	270	フェニル酢酸イソブチル	15	31	30	24	0.0005			
香料	271	フェニル酢酸エチル	15	304	300	240	0.0051			
香料	272	フェノールエーテル類	15	11,988	12,000	9,600	0.205			
香料	273	フェノール類	15	636	650	520	0.011			
酸化防止剤	275	ブチルヒドロキシシアニソール	7	150,000	20,000	16,000	0.340	25	1.36	
有機酸類（酸味料）	276	フマル酸	17	2,400,000	2,000,000	1,600,000	34.2			34)
有機酸類（調味料）	277	フマル酸一ナトリウム	17	410,000	410,000	328,000	7.10			34)
香料	278	フルフラール及びその誘導体	15	1,133	2,000	1,600	0.030	25	0.12	
保存料	280	プロピオン酸	4	6,300	6,300	5,000	〔1.88〕	制限せず		35)
香料	281	プロピオン酸イソアミル	15	920	920	736	0.016			
香料	282	プロピオン酸エチル	15	32,663	33,000	26,400	0.565			
保存料	283	プロピオン酸カルシウム	4	72,000	72,000	57,600				35)
保存料	284	プロピオン酸ナトリウム	4	60,000	60,000	48,000				35)
香料	285	プロピオン酸ベンジル	15	276	300	240	0.0051			
その他用途添加物（溶剤）	286	プロピレングリコール	16	15,188,570	2,700,000	2,160,000	46.2	1,250	3.7	
乳化剤	287	プロピレングリコール脂肪酸エステル	12	1,100,000	1,100,000	880,000	18.8	1,250	1.5	
香料	288	ヘキサン酸	15	4,528	4,500	3,600	0.077			
香料	289	ヘキサン酸アリル	15	16,125	16,000	12,800	0.274	6.5	4.2	
香料	290	ヘキサン酸エチル	15	10,801	10,000	8,000	0.171			

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香料	291	ヘプタン酸エチル	15	538	600	480	0.010	125	0.008	
香料	292	l-ペリルアルデヒド	15	4,001	4,500	3,600	0.077			
香料	293	ベンジルアルコール	15	48,313	48,000	38,400	0.822	250	0.329	
香料	294	ベンズアルデヒド	15	3,170	3,200	2,560	0.055	250	0.022	
香料	295	芳香族アルコール類	15	13,227	13,300	10,640	0.230			
香料	296	芳香族アルデヒド類	15	2,582	3,000	2,400	0.051			
酸化防止剤	297	没食子酸プロピル	7	1,000	2,000	1,600	0.034	70	0.05	
糊料	298	ポリアクリル酸ナトリウム	6	536,000	20,000	16,000	0.340			
ガムベース	299	ポリイソブチレン	10	560,000	730,000	0	0			
糊料	300	ポリビニルポリピロリドン	6	280,000	280,000	0	0.000			
ガムベース	301	ポリブテン	10	80,000	110,000	0	0			
無機化合物（リン酸化合物）	302	ポリリン酸カリウム	19	51,000	20,000	16,000	0.340	Pとして3,500		
無機化合物（リン酸化合物）	303	ポリリン酸ナトリウム	19	3,462,000	1,800,000	1,440,000	30.8	Pとして3,500		
香料	304	d-ボルネオール	15	3,440	3,400	2,720	0.058			
香料	305	マルトール	15	159,044	160,000	128,000	2.740	50	5.48	
甘味料	306	D-マンニトール	1	412,066	380,000	304,000	6.50	特定せず	—	
無機化合物（リン酸化合物）	307	メタリン酸カリウム	19	36,000	10,000	8,000	0.17	Pとして3,500		
無機化合物（リン酸化合物）	308	メタリン酸ナトリウム	19	1,220,000	900,000	720,000	15.4	Pとして3,500		
強化剤（アミノ酸系）	309	DL-メチオニン	13	10,800	10,800	8,600	0.18			
強化剤（アミノ酸系）	310	L-メチオニン	13	1,500	1,500	1,200	0.026			
香料	311	N-メチルアントラニル酸メチル	15	2,150	2,200	1,760	0.038	10	0.38	
糊料	312	メチルセルロース	6	20,000	20,000	16,000	0.340	特定せず		
香料	313	メチルβ-ナフチルケトン	15	103	100	80	0.002			
強化剤	314	メチルヘスペリジン	14	11,200	11,200	6,272	0.134			
香料	315	dl-メントール	15	3,277	3,300	2,640	0.057	200	0.029	
香料	316	l-メントール	15	153,431	200,000	160,000	3.42	200	1.73	
その他用途添加物（被膜剤）	317	モルホリン脂肪酸塩	16	5,100	3,600	0	0	—	—	
強化剤	318	葉酸	14	5,400	1,500	840	0.018			
香料	319	酪酸	15	13,778	14,000	11,200	0.240			
香料	320	酪酸イソアミル	15	7,267	7,000	5,600	0.120	150	0.08	
香料	321	酪酸エチル	15	30,382	30,000	24,000	0.510	750	0.068	

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香料	322	酪酸シクロヘキシル	15	120	120	96	0.002			
香料	323	酪酸ブチル	15	738	740	592	0.013			
香料	324	ラクトン類	15	44,019	44,000	35,200	0.753			
強化剤（アミノ酸系）	325	L-リジンL-アスパラギン酸塩	13	0	0	0	0			
強化剤（アミノ酸系）	326	L-リジン塩酸塩	13	336,000	336,000	269,000	5.76			
強化剤（アミノ酸系）	327	L-リジンL-グルタミン酸塩	13	0	0	0	0			
香料	328	リナロオール	15	4,024	4,000	3,200	0.068	25	0.272	
調味料	329	5'-リボヌクレオチドカルシウム	11	38,000	38,000	30,400	0.65	特定せず	−	
調味料	330	5'-リボヌクレオチド二ナトリウム	11	1,658,920	1,630,000	1,304,000	27.1	特定せず	−	
強化剤（ビタミンB2）	331	リボフラビン	14	22,800	22,800	12,768	0.273			
強化剤（ビタミンB2）	332	リボフラビン酪酸エステル	14	200	200	69	0.0015	※着色料と合せて，合成リボフラビン等のGroup ADI		
強化剤（ビタミンB2）	333	リボフラビン5'-リン酸エステルナトリウム	14	8,600	8,600	3,777	0.081	〔25〕※	〔1.42〕	
無機化合物（酸アルカリ）	334	硫酸	20	3,400,000	4,500,000	0	0			
無機化合物（ミョウバン）	335	硫酸アルミニウムアンモニウム	21	280,000	225,000	180,000	3.90			36,37)
無機化合物（ミョウバン）	336	硫酸アルミニウムカリウム	21	2,155,380	1,970,000	1,576,000	33.7	350	7.8	36,37)
無機化合物（その他）	337	硫酸アンモニウム	22	53,000	53,000	42,400	0.91			
無機化合物（カルシウム剤）	338	硫酸カルシウム	18	4,467,000	6,500,000	3,400,000	72.8	特定せず		
無機化合物（その他）	339	硫酸第一鉄（乾燥）	22	0	0	0	0			
無機化合物（その他）	339	〃 （結晶）	22	39,000	39,000	31,200	0.668			
無機化合物（その他）	340	硫酸ナトリウム	22	138,800	138,800	111,040	2.38	特定せず		38)
無機化合物（その他）	341	硫酸マグネシウム	22	1,345,600	1,345,600	1,076,480	23			39)
有機酸類（酸味料）	342	DL-リンゴ酸	17	4,200,000	3,300,000	2,640,000	56.5			40)
有機酸類（調味料）	343	DL-リンゴ酸ナトリウム	17	925,000	925,000	740,000	15.8			40)
無機化合物（酸アルカリ）	344	リン酸	20	1,700,000	1,870,000	520,000	11.23			33)
無機化合物（リン酸化合物）	345	リン酸三カリウム	19	322,000	300,000	240,000	5.14			33)
無機化合物（カルシウム剤）	346	リン酸三カルシウム	18	436,000	440,000	352,000	6.85	Pとして3,500	0.02	33)
無機化合物（リン酸化合物）	347	リン酸三マグネシウム	19	166,000	160,000	128,000	2.74	Pとして3,500		33)
無機化合物（リン酸化合物）	348	リン酸水素二アンモニウム	19	437,000	30,000	24,000	0.510	Pとして3,500		33)
無機化合物（リン酸化合物）	349	リン酸二水素アンモニウム	19	158,000	20,000	16,000	0.340	Pとして3,500		33)
無機化合物（リン酸化合物）	350	リン酸水素二カリウム	19	868,000	700,000	560,000	12.00	Pとして3,500		33)
無機化合物（リン酸化合物）	351	リン酸二水素カリウム	19	572,000	850,000	680,000	14.55	Pとして3,500		33)

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無機化合物（カルシウム剤）	352	リン酸一水素カルシウム	18	110,000	110,000	88,000	1.88	Pとして3,500	0.05	33)
無機化合物（カルシウム剤）	353	リン酸二水素カルシウム	18	464,000	460,000	368,000	7.88	Pとして3,500	0.23	33)
無機化合物（リン酸化合物）	354	リン酸水素二ナトリウム（無水）	19	1,581,000	600,000	480,000	10.30	Pとして3,500		33)
無機化合物（リン酸化合物）	355	リン酸二水素ナトリウム（無水）	19	649,000	300,000	240,000	5.14	Pとして3,500		33)
無機化合物（リン酸化合物）	356	リン酸三ナトリウム（無水）	19	978,000	350,000	280,000	5.99	Pとして3,500		33)
				1,831,361,789	742,191,657	304,288,400	6,542			

表3－1，表3－2　および表4－2についての脚注

1）ADIは1999年6月開催の第53回JECFAまでの数値を採用
　NS＝（not specified）ADIを特定せず　　NL＝（not limited）ADIを制限せず
2）摂取量，ADI共に亜硝酸として
3）摂取量はアスコルビン酸として
4）摂取量，ADI共に二酸化硫黄として，亜硫酸化合物のグループADI
5）摂取量，ADI共に25，26を合わせ安息香酸として
6）52，53を合わせて，エリソルビン酸として
7）食品使用量，摂取量ともクエン酸（無水）として
8）80～85を含め食品使用量，摂取量ともクエン酸（無水）として
9）ADI値，但しポリグリセリン脂肪酸エステル，縮合リシノレイン酸のADIは，それぞれ別に25，7.5 mg/kg/日と定められている。
10）91，92合わせてグルコン酸として
11）1-1，93，94，214-1を含めてグルコン酸として
12）109，110合わせてコハク酸として
13）食品使用量$V.D_3$として8.4単位
14）摂取量は氷酢酸として
15）食品への直接使用量は有効塩素4％として
16）食品使用量は85％物として。摂取量は二酸化硫黄として0.97
17）酒石酸としてのグループADI
18）食品使用量，摂取量，ADIは酒石酸としてのグループADI
19）摂取量，ADIは二酸化窒素として
20）公定書下限値を基に，レーキ色素の10％を原色素とし，タール色素と合計した数値
21）185，186を合わせてソルビン酸として
22）摂取量はデヒドロ酢酸として
23）輸入食品よりの推定摂取量を含む
24）100％として
25）摂取量，232～234合わせて乳酸として
26）60％液
27）236，237合わせて，アナトーエクストラクトとして
28）239～243の合算。摂取量はパラオキシ安息香酸として
29）ビタミンA油として（1g中にビタミンA1,000,000単位として計算した量）
30）一日摂取量はビタミンA油として（152単位に相当）
31）ピリドキシンとして
32）摂取量，ADI二酸化硫黄として，亜硫酸化合物のグループADI
33）ADIはMTDI（最大耐用一日摂取量）リンとして全てのリン摂取源からの総量
34）フマル酸として

35) 280, 283, 284合わせて，摂取量はプロピオン酸として
36) 335, 336合わせて乾燥物として
37) ADI はアルミニウムとして，PTWI
38) 無水物として
39) 3水塩として
40) リンゴ酸として

以　上

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Analysis of residual solvent in thickeners by headspace gas chromatography using a standard addition method

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Chiye Tatebe, Hiromi Kawasaki, Hiroki Kubota, Kyoko Sato, Ken-ichi Tanamoto, Yoko Kawamura

Division of Food Additives, National Institute of Health Sciences

Abstract

Headspace gas chromatography (HS-GC) is an accepted method for analysis of residual solvents in pharmaceuticals, food additives and food. The amounts of residual solvent present in various food thickeners were analysed by HS-GC standard addition method. Conditions for the HS-GC were optimised, and equilibration time was determined to be 40 min at 60°C for the determination of residual solvent. The results were very similar to those obtained by distillation and gas chromatography (Distillation–GC). We conclude that both methods are equally efficient for the determination of residual solvent in thickeners. In addition capillary column (Aquatic-2 GL Sciences Co.) was used to analyze by headspace or distillation.

Keywords : residual solvent, food additive, headspace gas chromatography (HS-GC), standard addition method

I Introduction

The production of natural food additives often involves the use of organic solvents. The eighth edition of Japan's Specifications and Standards for Food Additives[1], restricts the types of organic solvents that can be used in the manufacturing of food additives—extracts and colours—and regulates the levels of residual solvents in these food additives.

The level of residual solvents in food additives in Japan including thickeners and stabilizers, such as carob bean gum, xanthan gum, guar gum, gellan gum, purified carrageenan, pectin, processed eucheuma algae, macrophomopsis gum and rhamsan gum, need to be determined to ensure international consistency with the Joint FAO/WHO Expert Committee on Food Additives (JECFA) standards. In the eighth edition of Japan's Specifications and Standards for Food Additives, the standard residual solvent levels for 2-propanol or 2-propanol plus methanol were set for nine different thickeners.

There are several methods available for the quantification of residual solvents in thickeners. Distillation with water is the most widely used for collecting residual solvents from thickeners, and this is the method adopted in the eighth edition of Japan's Specifications and Standards for Food Additives. This method uses gas chromatography (GC) with a packed column, because the distillate includes large amounts of water. Headspace (HS)-GC using packed columns is also the method of choice for purity testing, in which the residual solvent is converted into corresponding nitrite esters, according to the JECFA monograph for carob bean gum and guar gum*1. At present, HS-GC using capillary columns for determining levels of volatile substances is used in laboratories because of its high resolution. The HS-GC method is widely used to determine volatile substances in pharmaceutical drugs[2], and the standard addition method primarily helps avoid influences of the sample matrix[3]. Comparability between HS-GC using the standard addition method and distillation-GC has not been assessed. Moreover, the eighth edition of Japan's Specifications and Standards for Food Additives and JECFA have not established the use of capillary columns for detecting residual solvents in thickeners. It would be desirable to have information on the usefulness of capillary columns when analysing samples containing large amounts of water with gas chromatography.

Corresponding author: Chiye Tatebe, Division of Food Additives, National Institute of Health Sciences, 1-18-1, Kami-yoga, Tokyo 158-8501, Japan

*1 JECFA (Joint FAO/WHO Expert Committee on Food Additives), Online Edition: "Combined Compendium of Food Additive Specifications" http://www.fao.org/ag/agn/jecfa-additives/search.html?lang=en

This study determines residual solvents in food thickeners such as carob bean gum, guar gum and gellan gum by HS-GC, using the standard addition method and distillation-GC. We compared the results of both methods, and investigated the use of capillary columns for determining residual solvents in thickeners.

II Materials and methods

1. Chemicals and instruments

Methanol (HPLC grade) was obtained from Merck (Damstadt, Germany), 2-propanol (HPLC grade), tertiary-butanol (*tert*-butanol), guar gum, carob bean gum, and gellan gum were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Headspace vials (10 ml) and aluminium crimp seals with PTFE septa, an Aquatic-2 capillary column (60 m × 0.25 mm i.d., 1.4 μm film thickness) and a glass column (2.1 m × 3.2 mm i.d.) packed with Gaskuropak54 60/80 were supplied by GL sciences Inc. (Tokyo, Japan). Gas chromatography (HP 5890) using a flame ionisation detector (FID) and headspace sampler (HP 7694) for the standard addition method were supplied by Hewlett Packard Inc.. Gas chromatography using a FID (GC 14B) for the distillation method was supplied by Shimadzu Corp. (Kyoto, Japan).

2. Solvent additional sample preparation

The regulation of residual solvent limits 2-propanol to 1% in carob bean gum and guar gum, to 0.075% in gellan gum in the eighth edition of Japan's Specifications and Standards for Food Additives. Because there are no residual solvents in commercial versions of these gums, 2-propanol was added to guar gum, carob bean gum, and gellan gum. Since methanol was limited in other some thickeners, methanol was added to them to obtain additional information. A hundred millilitres of methanol and 2-propanol were added to 200 g of each gum. The gums were placed in a fume cupboard for a week at room temperature. The gums were then sealed in glass bottles and refrigerated at 4°C.

3. Standard solutions for the standard addition method of HS-GC

Using water, 0.8 g each of methanol and 2-propanol were made up to 100 ml, 1 ml of each resultant solution was made up to 100 ml in water as standard A (Std A: 80 μg/ml), of which 10 and 5 ml were diluted to 20 ml in water as standard B (Std B: 40 μg/ml) and C (Std C: 20 μg/ml), respectively. In the case of gellan gum, the amount of 2-propanol present was so high that 10 times the concentration of the standard solutions had to be used.

4. Sample preparation for HS-GC

Exactly 0.04 g of the gum was weighed out in four 10 ml vials, and a magnetic stirring bar and 5 ml of either water, Std A, Std B or Std C were added to each vial. Then the vials were immediately sealed using an aluminium cap with a PTFE septum. The vials were left standing overnight at room temperature. After stirring the solution in the vials for 1 min, the vials were then placed in the headspace sampler.

5. Procedure for HS-GC

Helium served as the carrier gas at 250 kPa (1.5 ml/min). The oven in the GC was maintained at 40°C for 6 min, and then heated to 110°C at a rate of 4°C/min. Finally, the temperature was increased to 250°C at a rate of 25°C/min, and maintained for 10 min. The injection temperature was 200°C, and the detector temperature was 250°C.

The headspace in the vial was injected as follow: the vials were kept at 60°C for 40 min, then pressurised for 0.15 min with carrier gas, the sample loop temperature was adjusted to 110°C, and the transfer line temperature was 130°C. Headspace gas was introduced to the GC column in the split mode in a 10:1 or 90:1 ratio.

Quantification was performed by the standard addition method. The peak areas of methanol and 2-propanol were measured. The area of methanol or 2-propanol for each sample (one spiked with water and three spiked with different levels of standard solutions) was plotted on the y-axis and the concentration (μg/g) of solvent in the standard solution was plotted on the x-axis. A calibration curve was obtained by the simple linear least squares analysis method. The absolute x-intercept value at $y = 0$ represents the concentration of the solvent as C_{sa} (μg/g).

6. Sample preparation for distillation and GC chromatography

Tert-butanol (0.1 g) was dissolved in 100 ml of water as the internal standard solution. Methanol and 2-propanol (0.5 g) each were weighed out exactly and made up to 50 ml with water, of which 5 ml was diluted to 50 ml using water. 2 ml of the methanol and 2-propanol mixture solution, and 4 ml of internal standard solution, were made up to 100 ml with water as standard solution.

The gums were weighed out (2 g each) in 300 ml round flasks, and 200 ml of water, 5 ml of silicon oil and a few boiling stones were added and mixed. The flasks were then connected to a distillation apparatus. The flasks were heated using a heater, and when the water and gum started to boil, the temperature of the heater was reduced, to keep the mixture boiling without boiling over the connection to the distillation apparatus. The internal standard solution (4 ml) was added to a 100 ml volumetric flask. Distillate was added at a rate

of 2–3 ml/min to a 100 ml volumetric flask. The process of distillation was stopped when 90 ml of distillate was collected, and the distillate was made up to 100 ml by adding water.

GC was performed using a Shimadzu GC-14B equipped Aquatic-2 capillary column, with helium as a carrier gas at 250 kPa (1.0 ml/min). The oven in the GC was maintained at 40°C for 6 min, and then the temperature was increased to 110°C at a rate of 4°C/min. Finally, the temperature was increased to 250°C at a rate of 25°C/min, and maintained for 10 min. The injection temperature was 200°C, and the detector temperature was 250°C. The injection volume was 1 µl, and the injection was performed in split mode in a 100:1 ratio.

One microliter of the standard solution and distillate were injected into the GC with FID, and the areas under the peak for *tert*-butanol, methanol and 2-propanol were measured. The amount of 2-propanol and methanol was determined by the ratio of the area of methanol or 2-propanol to that of *tert*-butanol. The ratios of standard solution and distillate were denoted as Q_S and Q_T. Q_S and Q_T were calculated using the formula A_a/A_{TBA}, in which A_a is the area of each alcohol peak (a = methanol, 2-propanol), and A_{TBA} is the area of the *tert*-butanol.

The percentage of solvent was calculated as the weight of solvent (g)/weight of gum (g) × Q_T/Q_S × 0.4.

7. Comparison between packed column and capillary column with FID (GC 14B)

A standard solution of methanol, 2-propanol and *tert*-butanol for distillation was applied in a packed or capillary column with FID (GC 14B) and the peaks compared.

The packed column was equipped with GC 14B; the column temperature was 120°C; the injection port temperature was 200°C; the carrier was helium flowing at 250 kPa. The GC conditions, using the capillary column with GC 14 B, were the same as for the distillation method.

8. Recovery tests with spiked sample preparation by HS-GC

To evaluate the repeatability and the accuracy of the HS-GC, recovery tests were carried out by analyzing two different concentration levels of 2-propanol with 1 and 0.5% for carob bean gum and guar gum, or 0.075 and 0.0375% for gellan gum. Exactly 0.04 g of the gum was weighed out in four 10 ml vials, and a magnetic stirring bar and 5 ml of either water, Std A, Std B or Std C were added to each vial. In case of gellan gum Std A, Std B and Std C were diluted into 6, 3 and 1.5 µg/ml, respectively. Fifty microliter of standard solution containing 2-propanol with suitable concentration was added to all sample preparations before sealing the vials. The spiked samples were treated according to the sample preparation procedure for HS-GC.

Ⅲ Results and Discussion

1. Distillation-GC using capillary column

Figure 1 shows gas chromatograms of the standard solutions of methanol, 2-propanol and *tert*-butanol in water taken with GC-FID using packed and capillary columns.

In the chromatogram of the packed column (Fig. 1 a), methanol eluted at ca. 2.3 min, 2-propanol at 12.2 min and *tert*-butanol at 23.4 min. Although these peaks were clear, the methanol peak appeared near the injection artefact at around 2 min, and the peak of *tert*-butanol was broad. On the other hand, in the chromatogram of the capillary column (Fig. 1 b), methanol eluted at ca. 4.2 min, 2-propanol at 6.8 min and *tert*-butanol at 7.4 min. All the peaks were clear and the area of each peak was not spread out in this chromatogram. There was no degradation of the capillary column for repeated injections of the sample solutions. These results show that the method can accurately analyse distillate containing water from thickeners.

Carob bean gum, guar gum and gellan gum were distilled with water, and the distillate collected and analysed by GC-FID using a capillary column. Figure 2 shows the chromatogram of the distillate from guar gum, carob bean gum, and gellan gum analysed by GC-FID using a capillary column. No significant difference was observed in the area of the internal standard (*tert*-butanol), and the peak showed good resolution.

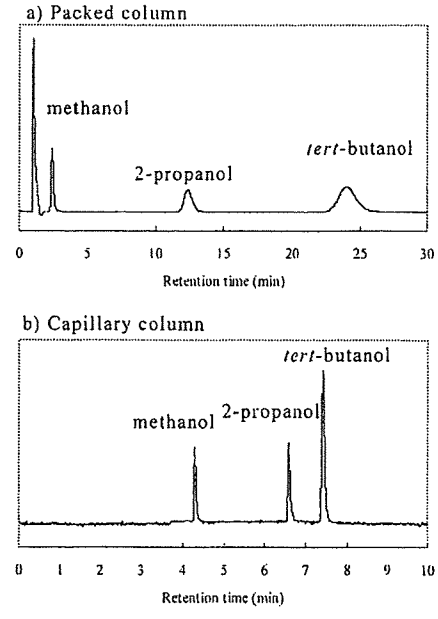

Fig. 1. GC-FID Chromatogram of methanol, 2-propanol and *tert*-butanol in water using a) a packed column, and b) a capillary column

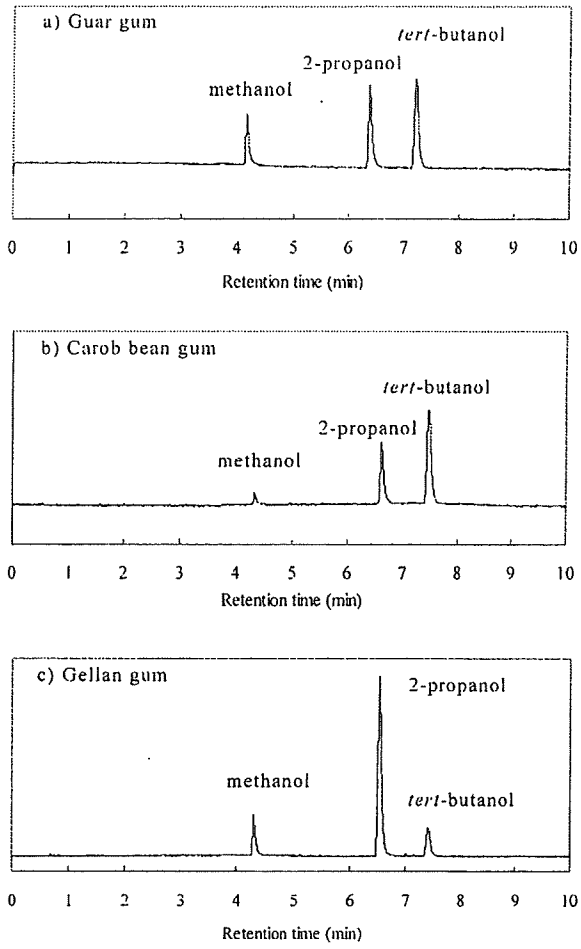

Fig. 2. GC-FID chromatogram of residual solvent in spiked a) guar gum, b) carob bean gum and c) gellan gum after distillation

2. Headspace Gas Chromatography (HS-GC)

The sample vials were kept for various pre-incubation times (5 min to overnight) at various temperatures (room temperature to 50°C), and HS-GC was performed (vial equilibration at 60°C for 40 min). The results all showed almost the same or less methanol and 2-propanol than obtained by distillation, but the amount of methanol varied for a short pre-incubation time or at a low equilibration temperature.

This variability was caused by incomplete swelling of the gums and equilibration in the vials. Therefore, we kept the sample vials overnight at room temperature. Furthermore, the peaks of methanol and 2-propanol widened, depending on the equilibration temperature for the headspace vials in the headspace sampler. To improve the precision of the analysis, we performed the analysis at an equilibration temperature of 70°C, but the residual solvent value was below that seen at 60°C.

The residual solvents absorbed in the thickener are released by swelling the thickener in water. Guar gum, carob bean gum and gellan gum were swollen in water or the standard solution in sealed vials overnight at room temperature. We used the standard addition method to avoid the affect of the sample matrix, because each thickener shows different viscous behaviour in water. Figure 3 shows the chromatogram of the HS-GC of the residual solvent in each sample. Methanol and 2-propanol eluted at ca. 5.4 min and ca. 8.1 min, respectively. These peaks were completely resolved from the other volatiles.

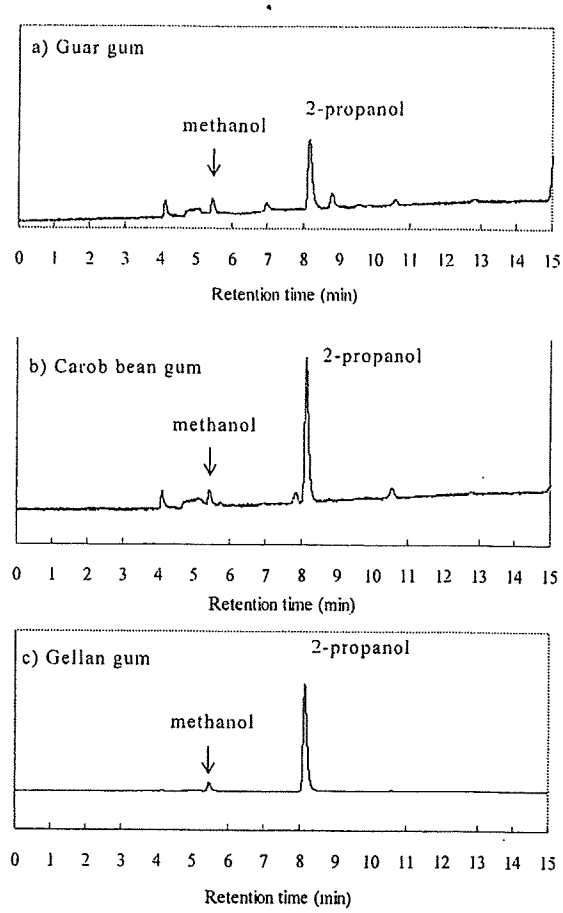

Fig. 3. HS-GC chromatogram of residual solvent in spiked a) guar gum, b) carob bean gum and c) gellan gum after keeping them overnight at room temperature HS oven temperature: 60°C, equilibration time: 40 min.

3. Quantification of methanol and 2-propanol by HS-GC and distillation-GC

Table 1 shows the quantitative values of methanol and 2-propanol in carob bean gum, guar gum and gellan gum analysed by HS-GC and distillation. The values obtained by HS-GC were similar to those obtained by distillation-GC for all the gums, however, the values of 2-propanol by distillation of carob bean gum and gellan gum were slightly higher than those by HS-GC. The values of 2-propanol in gellan gum were

spread out, probably due to overload of the capillary column. The differences in values were not statistically significant. From Table 1, both methods obtained similar quantitative values and the distillation-GC and HS-GC methods showed similar effectiveness in analysing the residual solvent in thickeners.

HS-GC is widely used for determining residual solvents in pharmaceutical substances, and is also used for some natural food additives and flavours[4-6], but the method has not yet been introduced in Japan's Specification and Standard for Food Additives. When HS-GC is used for quantitative determination of residual solvents, the sample must be well dissolved or suspended uniformly to volatilise the residual solvents. However, coexisting matrices in the sample affect the partial coefficient between the gaseous and sample phases in the HS vial because of their stickiness[3]. To avoid this affect of the matrix, we used the standard addition for the quantitative analysis of residual solvents in thickeners by HS-GC.

Table 1. Comparison of the quantitative values obtained by Distillation-GC and HS-GC methods

		Content (%)*	
		GC-FID	
		HS	
Sample		60°C, 40 min	Distillation
Guar gum (add)	Methanol	0.11 ± 0.02	0.13 ± 0.01
	2-propanol	0.21 ± 0.02	0.20 ± 0.01
Carob bean gum (add)	Methanol	0.02 ± 0.01	0.03 ± 0.00
	2-propanol	0.11 ± 0.02	0.13 ± 0.02
Gellan gum (add)	Methanol	0.33 ± 0.02	0.30 ± 0.02
	2-propanol	1.30 ± 0.14	1.31 ± 0.03

* Each value is mean ± S.D. of 3 trials

4. Recovery tests with spiked samples by HS-GC

The repeatability and accuracy of HS-GC were evaluated by a recovery test with each gum. Table 2 shows the recoveries of spiked 2-propanol from carob bean gum, guar gum and gellan gum. The recoveries were 83.1~107.5%, and the relative standard deviations (RSD) were 2.9~9.6%. RSD(%) at a spiked level of 1% were improved by modification of the GC injector split ratio from 10:1 to 90:1

The thickeners used in this study were insoluble in organic solvents, but soluble in water. However, adding a small amount of water made them into a hard gel, which made HS-GC analysis difficult. Because adding even 5 ml of water or Std solution to 0.04 g of thickeners is not enough to eliminate specific viscous behaviours, we tried to avoid the matrix effect by using the standard addition method in this study.

Table 2. Recovery tests of 2-propanol in sample preparations

Sample	Spiked level (%)	Recovery (%)[a]	RSD (%)
Carob bean gum	1	106.9	5.8
	0.5	83.1	9.3
Guar gum	1	103.7	9.6
	0.5	104.9	2.9
Gellan gum	0.075	107.5	7.2
	0.0375	106.9	6.2

[a] Mean of 3 replicates

Ⅳ Conclusions

Residual solvents in thickeners were determined by HS-GC using a standard addition method and the distillation-GC method. The results of both methods were very similar, and both methods were equally effective. The standard addition method is effective for determining the amount of residual solvent in viscous material. Capillary columns can be used to analyse the residual solvent containing water in thickeners in both HS-GC and distillation-GC methods, and there was no problem in separating the peaks of methanol and 2-propanol with good resolution.

Ⅴ Acknowledgement

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論　　文

標準添加法を用いたヘッドスペースガスクロマトグラフィー法による増粘安定剤中の残留溶媒の分析

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建部千絵、河﨑裕美、久保田浩樹、佐藤恭子、棚元憲一、河村葉子

国立医薬品食品衛生研究所　食品添加物部

キーワード：残留溶媒、食品添加物、ヘッドスペースガスクロマトグラフィー（HS-GC)、標準添加法

概　要

ヘッドスペースガスクロマトグラフィー（HS-GC法）は薬品、食品添加物および食品中の残留溶媒の分析法として頻繁に用いられている方法である。本研究では、増粘安定剤（カロブビーンガム、グアーガム、ジェランガム）中のメタノールおよび2-プロパノールを分析対象とし、標準添加法を用いたヘッドスペースガスクロマトグラフィー法（HS-GC法）および水で蒸留後、留液をガスクロマトグラフィーで分析する方法（蒸留-GC法）により分析し、両方法で得られる溶媒量の比較検討を行った。その結果、HS-GC法と蒸留-GC法ではほぼ同程度の溶媒量が検出された。以上の結果から、増粘安定剤のような粘性の高い試料に対して、標準添加法によるHS-GC法は蒸留-GC法などと同等有用な方法であると考えられた。また、いずれの分析法においてもキャピラリーカラム（Aquatic-2 ジーエルサイエンス（株））を用いて良好な分離が得られた。

連絡先：〒158-8501　東京都世田谷区上用賀1-18-1　国立医薬品食品衛生研究所食品添加物部　建部千絵

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

Shizuyo HORIYAMA,*[,a] Chie HONDA,[a] Kiyoko SUWA,[a] Yasuyo OKADA,[a] Masanori SEMMA,[a] Atsushi ICHIKAWA,[a] and Mitsuo TAKAYAMA[b]

[a] *School of Pharmacy and Pharmaceutical Sciences, Mukogawa Women's University; 11–68 Koshien-Kyuban-cho, Nishinomiya, Hyogo 663–8179, Japan: and* [b] *International Graduate School of Arts and Sciences, Yokohama City University; 22–2 Seto, Kanazawa-ku, Yokohama 236–0027, Japan.*

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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 μmol/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-

* To whom correspondence should be addressed. e-mail: horiyama@mukogawa-u.ac.jp

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 μm) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), TSKgel ODS-100V (particle size 3 μm) and TSKgel ODS-100Z (particle size 5 μm) were from TOSOH Co. (Tokyo, Japan), Hypersil GOLD aQ (particle size 3 μm) and Thermo Hypersil-Keystone BetaMax Neutral (4.6 mm i.d.×150 mm and 2.1 mm i.d.×150 mm; particle size 5 μm) were from Thermo Fisher Scientific K. K. (Yokohama, Japan). Carbon column Hypercarb (4.6 mm i.d.×150 mm; particle size 5 μm) 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 μm) 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 μm) 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 μl. 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 μl/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—3.39×10^{-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-

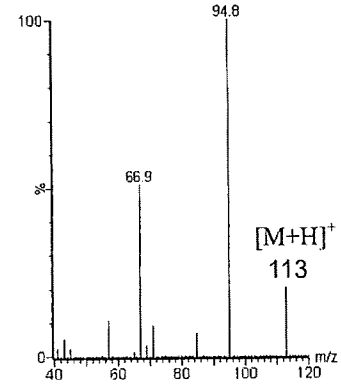

Fig. 1. Positive Ion Mode ESI Product Ion Spectra of $[M+H]^+$ *m/z* 113 with Optimized Collisional Energy

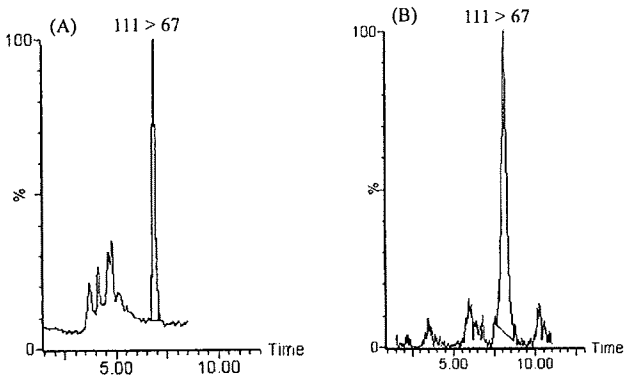

Fig. 2. Negative Ion Mode ESI SRM Chromatogram of Cytosol Fraction Spiked with SA

(A) An equal volume of SA 102.5 nmol/l was added to filtered cytosol, and 20 μl 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 μmol/l was added to filtered cytosol, and 20 μl was injected to LC/MS. Eluent: CH_3CN–H_2O (90 : 10), flow rate: 1 ml/min, column: Aqua Way Cation.

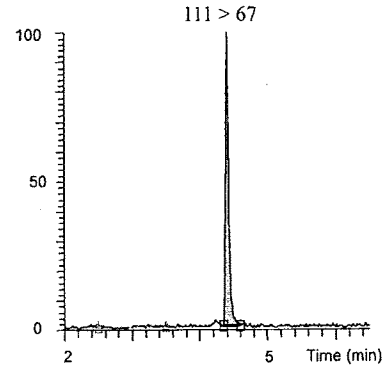

Fig. 3. Negative Ion Mode ESI High Resolution SRM Chromatogram of Cytosol Fraction Spiked with SA

An equal volume of SA 102.5 nmol/l was added to filtered cytosol, and 5 μl 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.

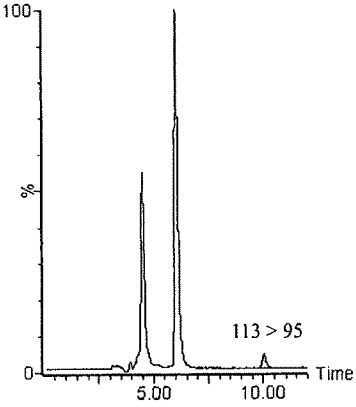

Fig. 4. Positive Ion Mode ESI SRM Chromatogram of Cytosol Fraction Spiked with SA

An equal volume of SA 102.5 nmol/l was added to filtered cytosol, and 5 μl 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 (l/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 μl) 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/5×10^6 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.

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「国際的動向を踏まえた食品添加物の規格、基準の向上に関する調査研究」
「食品添加物の規格基準の向上と摂取量に関する調査研究」

食品香料化合物の自主規格の作成に関わる調査研究

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研究者氏名　　長谷川　徳二郎