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Application Note

No.2

Multi-Residue Analysis of Pesticides in Green Tea Using Caffeine Removal Pretreatment

Y. Okamura

Food

1. Introduction

Green tea is becoming a popular beverage worldwide. Table 1-1 through Table 1-3 show the survey results for worldwide green tea production, and import and export quantities. With about 4 million tons produced worldwide, which is about half that of coffee bean production, China boasts the greatest rate of green tea production, followed by India, Kenya and Sri Lanka.

Sri Lanka is the greatest exporter of green tea, followed by Kenya, India and China. Domestic consumption is very high in India and China, with about 80 % of the production consumed in those countries. Japan also is a high-producing country, but due to even higher consumption, Japan also imports an amount which is equivalent to 50 % of its own production level.

The 27 European Union (EU) countries are the greatest importers, followed by the Russian Federation and the United Kingdom, with very high consumption clearly occurring in Europe.

Due to recent concern regarding food safety among consumers, advances in analytical methods for detecting and quantifying pesticide residues now permit the inspection of many crops for the presence of residual pesticides. With an increasing number of pesticides becoming subject to inspection every year, mass spectrometers are the instrument of choice for conducting simultaneous analyses targeting multiple pesticide residues.

The multi-residue analysis of pesticides in teas has become common worldwide. Caffeine, which is typically present in large quantities, can interfere with detection and quantitation of pesticides and other tea constituents, and is also a source of contamination in analytical instrumentation.

The development of an analytical method for multi-residue analysis of pesticides in green tea by gas chromatography with mass spectrometry (GCMS) is reported in this Application Note. A novel technique was employed to easily and efficiently eliminate caffeine to avoid any adverse effect on pesticide recoveries.

Table 1-1 Tea Production (2009)

Rank	Area	Production (tonnes)
1	China	1375780
2	India	972700
3	Kenya	314100
4	Sri Lanka	290000
5	Turkey	198601
6	Viet Nam	185700
7	Indonesia	146440
8	Japan	86000
9	Argentina	71715
10	Thailand	63707
11	Bangladesh	59500
12	Malawi	52559
13	Uganda	48663
14	Iran (Islamic Republic of)	165717
15	United Republic of Tanzania	32000
16	Myanmar	30500
17	Zimbabwe	20862
18	Rwanda	20000

Table 1-2 Tea Export (2009)

Rank	Area	Production (tonnes)
1	Sri Lanka	288528
2	Kenya	331594
3	China	305352
4	India	203863
5	EU(27)ex.int	29882
6	United Kingdom	27741
7	Germany	25301
8	Indonesia	92304
9	United Arab Emirates	23681
10	Viet Nam	82416
11	Malawi	47356
12	Belgium	7859
13	Argentina	69816
14	United Republic of Tanzania	30438
15	Russian Federation	9713
16	Netherlands	18158
17	Poland	8609
18	Uganda	44446

Table 1-3 Tea Import (2009)

Rank	Area	Production (tonnes)
1	EU(27)ex.int	249930
2	Russian Federation	182149
3	United Kingdom	145960
4	United States of America	110861
5	United Arab Emirates	75255
6	Egypt	80304
7	Pakistan	96932
8	Iran (Islamic Republic of)	51733
9	Japan	43301
10	Saudi Arabia	20331
11	Syrian Arab Republic	30651
12	Germany	44267
13	Canada	17353
14	France	17695
15	Poland	41784
16	Morocco	54400
17	Ukraine	26915
18	Netherlands	29982

Reference : Food and Agriculture Organization of The United Nations , FAOSTAT
http://faostat.fao.org/default.aspx

2. Maximum Pesticide Residue Levels in Tea and Analytical Method

The levels of pesticide residues in food are established in various countries around the world using Maximum Residue Levels (MRL) and Tolerance values. Methods of regulation vary depending on the country, but the applicable pesticides are recorded, their appropriate usage conditions are specified, and their MRL values are set for foods. Although these have been determined based on impact assessments on the human body, reference values are set in consideration of the various types of food products, as intake varies depending on the type of food.
Reference values are set by the EU and Japan for many pesticides used in tea production. In the EU, MRL are specified for each agricultural product under Regulation (EC) No. 396/2005 Annexes. In Japan, the Ministry of Health, Labour and Welfare establishes MRL values for each product, and these can be viewed on the Ministry's home page.
The analytical method follows the Official Method of the AOAC INTERNATIONAL (AOAC). The United States Food and Drug Administration (FDA) publishes the Pesticide Analytical Manual (PAM), which specifies multi-residue methods as well as methods for individual pesticide compounds. The PAM multi-residue simultaneous analysis methods include methods for Non-fatty Foods and for Fatty Foods. Japan's Ministry of Health, Labour and Welfare categorizes the multi-residue analytical method based on whether the product is a cereal or a fruit, and a test method for tea is also indicated. Fig. 1 illustrates the analysis flow specified in the test method indicated by Japan's Ministry of Health, Labour and Welfare. In Japan, the system by which pesticides are regulated in foods is referred to as a "positive list system," which is referred to below as Japan's Positive List Test Method.
In this investigation, 250 target pesticide compounds were analyzed following Japan's Positive List Test Method, and using the Shimadzu GCMS QP-2010 Plus shown below. Fig. 2 shows GCMS chromatogram of a 1 mg/L (ppm) standard mixture of the 250 pesticides. Analytical conditions are shown in Table 2.

Regulation (EC) No 396/2005
http://ec.europa.eu/food/plant/protection/pesticides/community_legislation_en.htm

Pesticide Analytical Manual (PAM)
http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/PesticideAnalysisManualPAM/default.htm

Japan's Ministry of Health, Labour and Welfare
http://www.mhlw.go.jp/english/topics/foodsafety/positivelist060228/index.html

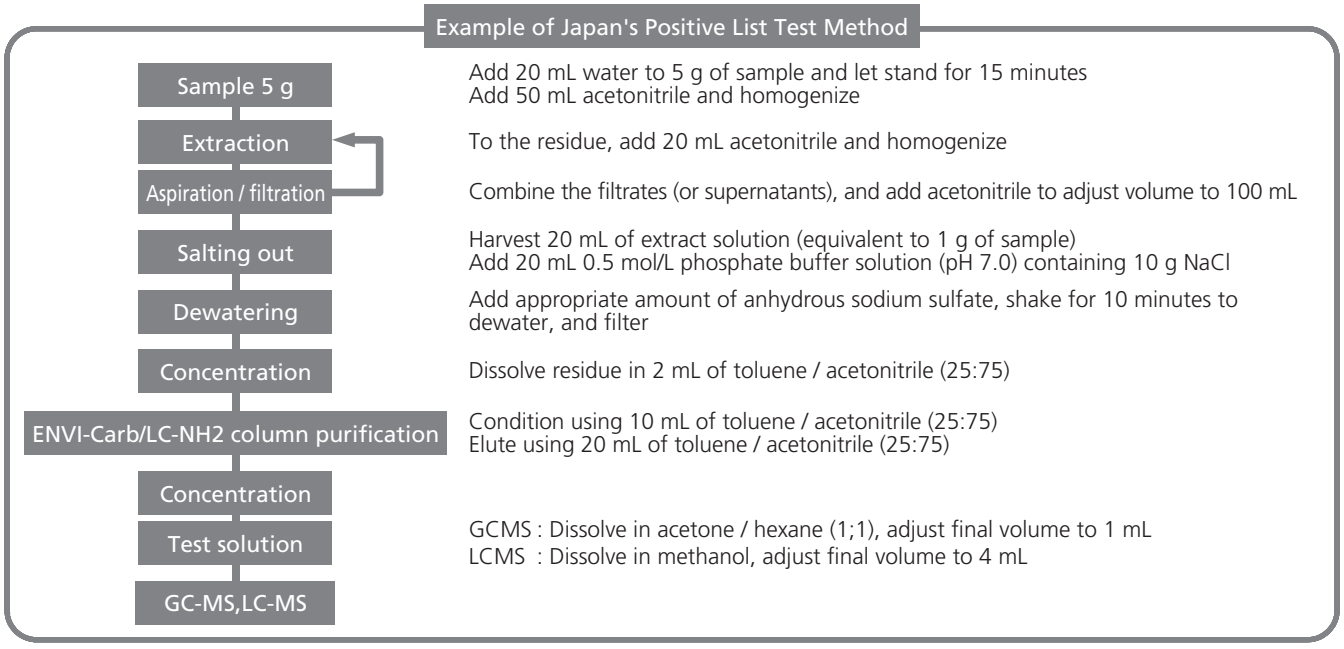


Fig. 1 Flow Diagram of Japan's Positive List Test Method

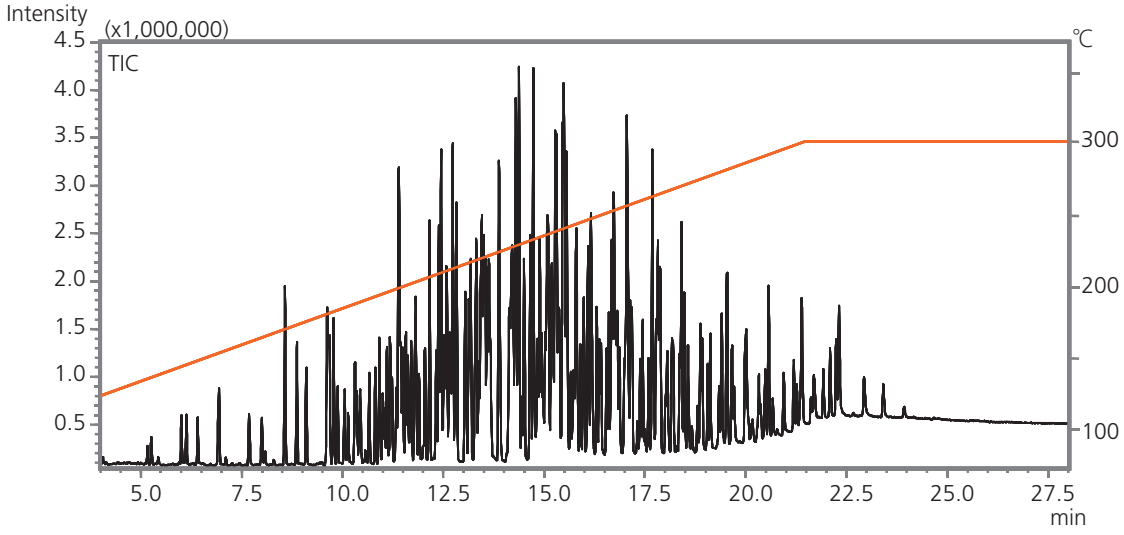


Fig. 2 Total Ion Chromatogram (TIC) of 250 Pesticides Analyzed by GCMS

Table 2 Analytical Conditions

Instrument	: Shimadzu GCMS-QP2010 Plus	Oven program	: 50 °C (1 minute) 25 °C/minute to 125 °C (0 minute) 10 °C/minute to 300 °C (10 minutes)
Inlet	: 1-µL injection volume High-pressure splitless mode (250 kPa, 1.5 minute) 250 °C	Interface	: 250 °C
		MS operation	: Electron Impact (EI) ionization Full scan mode, *m/z* 45-550
Column	: Rtx-5MS, 30 mL. × 0.25 mmI.D, df 0.25 µm Helium carrier gas, constant linear velocity (47.0 cm/second)		

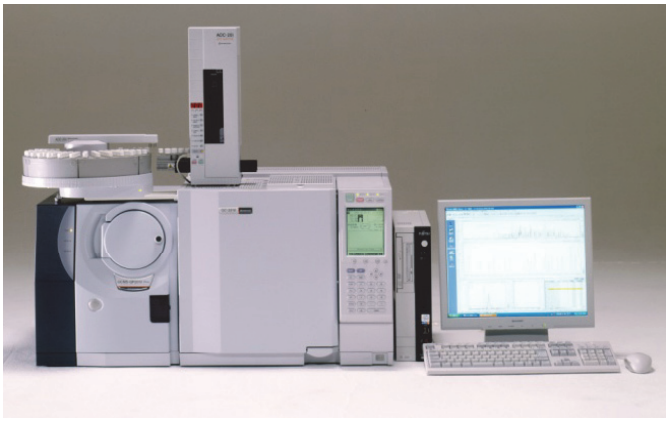
3. Analysis of Tea

Analysis of pesticide residues in commercially available green tea was conducted using the method conditions described above. After pretreatment according to Japan's Positive List Test Method, analysis by GCMS indicated that none of the 250 target pesticides were detected in the real-world sample. The Total Ion Chromatogram (TIC) obtained from analysis of the green tea extract is shown in Fig. 3. Ideally, method verification should be performed using a sample known to contain one or more of the target pesticides within the calibration range of the method. Since an actual tea sample contaminated with the target compounds was not available, a spike and recovery test was used to verify detection of pesticides and validate the method. A standard solution of pesticides was added to the green tea at a known concentration during the homogenization step. The extract was analyzed by GCMS and individual pesticide peaks were quantified against a calibration curve to verify recovery of the spiked pesticides.

The standard mixture of pesticides was added to the sample at a concentration of 100 µg/L (ppb).The recovery rate (%) obtained in this test was used to assess the test method. Table 3 shows the pesticides for which good recovery was obtained, at 70 % - 120 %.

Analytical interferences such as pigments, proteins, waxes, and other high molecular weight materials are co-extracted from the analytical sample along with the pesticides. Despite the absence of pesticide peaks in the chromatogram of Fig. 3, many peaks were detected. Because caffeine is present in large quantities in tea, its peak, which is seen to elute in the retention time range of 8 – 10 minutes, interferes with the detection of several pesticides in this portion of the chromatogram. Caffeine, which is present at high concentrations in coffee and various teas, including green tea and black tea, behaves much like the targeted pesticide compounds during extraction and cleanup, and is therefore difficult to eliminate using the pretreatment process that was used here.

Depending on the type of solid phase cartridge used as an extract clean-up step, caffeine can be retained, thereby allowing its elimination from the sample solution. Fig. 4 shows an example of caffeine reduction through the use of a Florisil® column. However, due to the similar characteristics of caffeine and pesticides, they are both likely to remain in the cartridge with this processing. Thus, a separate step would be required to elute the pesticides from the cartridge, which would increase the pretreatment time. It would also require two separate GCMS analyses for each sample of green tea. Thus, finding a simplified procedure for caffeine removal was a major priority with respect to the analysis of pesticide residues in tea.



Shimadzu GCMS-QP2010 Plus Used in This Study

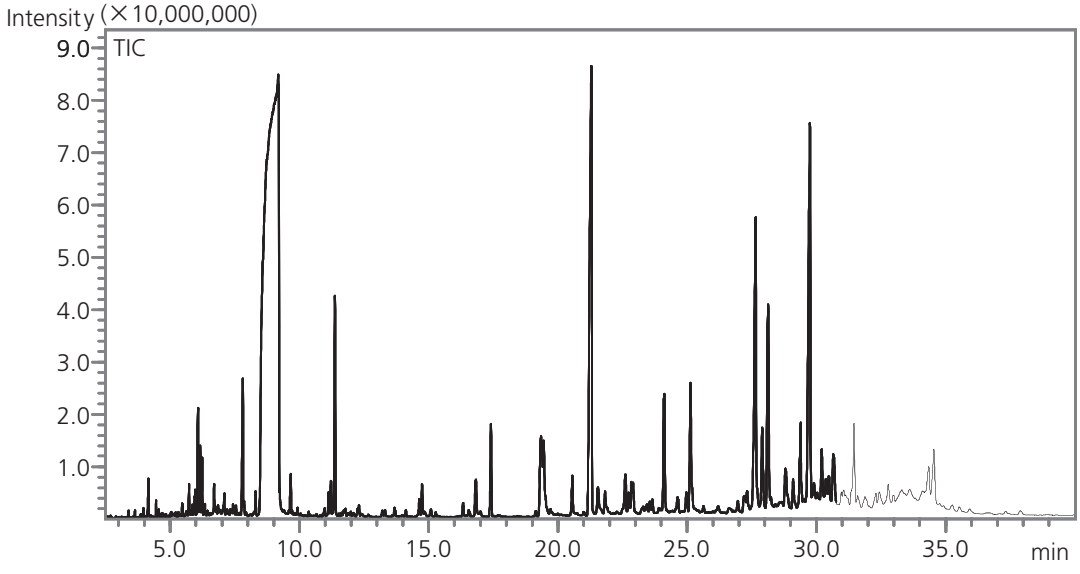


Fig. 3 GCMS Chromatogram of a Green Tea Extract

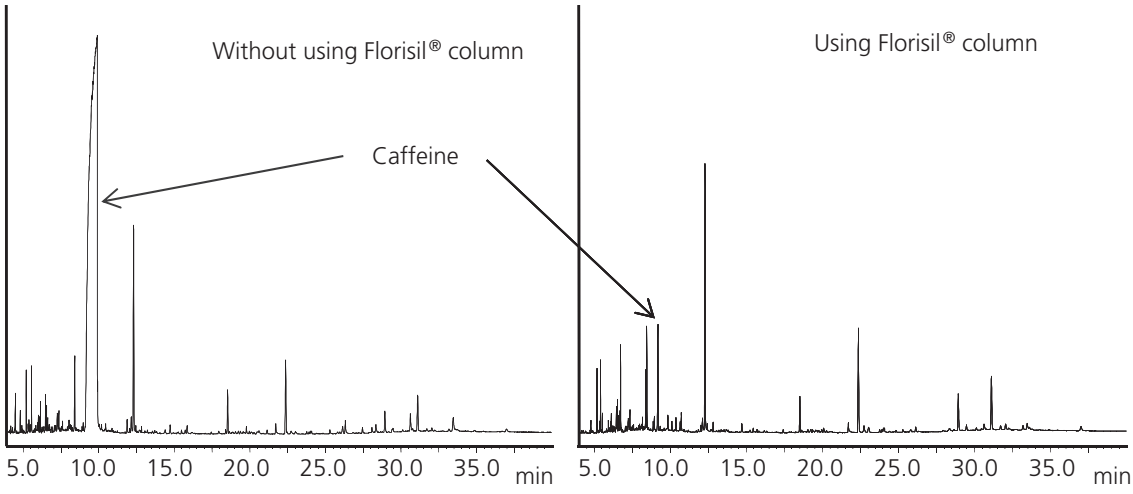


Fig. 4 Caffeine Reduction Due to Florisil® Column

Table 3 Green Tea Sample Spike and Recovery Test Results

Pesticide Name	Recovery (%)
EPTC	104
Mevinphos	86
Etridiazole	102
Chloroneb	97
XMC	106
Fenobucarb	88
Tecnazene	92
Propoxur	93
Propachlor	100
Diphenylamine	103
Ethoprophos	103
Chloropropham	103
Ethalfluralin	103
Trifluralin	113
Bendiocarb	92
Benfluralin	111
Cadusafos	100
alpha-BHC	99
Hexachlorobenzene	96
Dicloran	103
Dimethoate	90
Carbofuran	78
Atrazine	72
Propazine	79
beta-BHC	96
gamma-BHC	96
Propetamphos	101
Terbufos	101
Cyanophos	111
Quintozene	110
Pyroquilon	84
Pyrimethanil	93
Diazinone	100
Phosphamidon-1	73
Prohydrojasmon-1	103
Tefluthrin	102
delta-BHC	103
Triallate	109
Iprobenfos	111
Pirimicarb	90
Benoxacor	107
Benfuresate	111
Dichlofenthion	96
Propanil	89
Bromobutide	108
Spiroxamin-1	82
Acetochlor	102

Pesticide Name	Recovery (%)
Vinclozolin	93
Parathion-methyl	101
Chlorpyrifos-methyl	98
Tolclofos-methyl	107
Carbaryl	106
Alachlor	116
Heptachlor	75
Prometryn	100
Metalaxyl	86
Spiroxamin-2	89
Terbutryn	114
Malathion	108
Thiobencarb	111
Chlorpyrifos	109
Diethofencarb	110
Aldrin	92
Metolachlor	95
Fenpropimorph	98
Fenthion	104
(Z)-Dimethylvinphos	100
Parathion	120
Triadimefon	110
Isofenphos oxon	111
Chlorthal-dimethyl	109
Nitrothal-isopropyl	106
Bromophos	98
Fthalide	105
Diphenamid	91
Fosthiazate-2	99
E-Chlorfenvinphos	106
Dimethametryn	103
Penconazole	100
Heptachlor epoxide (A)	93
Oxy-Chlordane	96
(Z)-Pyrifenox	102
Heptachlor epoxide (B)	84
alpha-Chlorfenvinphos	117
Diclocymet-1	110
Quinalphos	100
Phenthoate	102
Zoxamide deg.	106
Procymidone	100
trans-Chlordane	105
Methidathion	70
Diclocymet-2	105
(E)-Pyrifenox	92
Tetrachlorvinphos	105

Pesticide Name	Recovery (%)
Fenamiphos	97
Flutolanil	114
Hexaconazole	113
Imazalil	92
Isoprothiolane	105
Profenofos	110
Tribufos	114
Pretilachlor	109
Uniconazole P	102
p,p'-DDE	104
Oxadiazon	113
Dieldrin	86
Oxyfluorfen	112
Flamprop-methyl	82
Myclobutanil	98
Buprofezin	84
Imibenconazole-debenzyl	70
Flusilazole	95
Thifluzamide	106
Bupirimate	88
Kresoxim-methyl	101
Isoxathion	117
Cyproconazole	92
Chlorfenapyr	115
Fenoxanil	115
Chlorobenzilate	106
beta-Endosulfan	91
Fensulfothion	94
(Z)-Pyriminobac-methyl	120
p,p'-DDD	107
o,p'-DDT	104
Mepronil	106
Fluacrypyrim	115
Triazophos	91
Benalaxyl	99
Edifenphos	87
Quinoxyfen	74
Propiconazole-1	104
Trifloxystrobin	115
Norflurazon	88
Lenacil	90
Endsulfan sulfate	91
p,p'-DDT	109
Propiconazole-2	95
(E)-Pyriminobac-methyl	103
Tebuconazole	97
Diclofop-methyl	115

Pesticide Name	Recovery (%)
Thenylchlor	111
Diflufenican	99
Propargite	101
Piperonyl butoxide	102
Zoxamide	70
Mefenpyl-diethyl	101
Iprodione	114
Pyridaphenthion	110
Bifenthrin	105
Bromopropylate	119
Phosmet	93
EPN	92
Tebufenpyrad	112
Bifenox	112
Anilofos	91
Phenothrin-2	102
Tetradifon	102
Phosalone	93
Pyriproxyfen	96
Cyhalothrin-1	104
Cyhalofop-butyl	100
Mefenacet	90
Cyhalothrin-2	120
Fenarimol	95
Pyrazophos	105
Pyraclofos	90
Fenoxaprop-ethyl	77
Bitertanol-1	89
trans-Permethrin	109
Bitertanol-2	101
cis-Permethrin	106
Pyridaben	97
Cyfluthrin-1	93
Cafenstrole	83
Fenbuconazole	88
Halfenprox	112
Flucythrinate-1	104
Flucythrinate-2	99
Fenvalerate-1	91
Fluvalinate-1	114
Fenvalerate-2	108
Fluvalinate-2	98
Difenoconazole-1	87
Difenoconazole-2	88
Flumiclorac-pentyl	96
Tolfenpyrad	93
Imibenconazole	91

4. Investigation of Caffeine Removal Prior to Detection of Pesticide Residues in Tea by GCMS

The presence of a large amount of caffeine in tea not only interferes with the detection of pesticides by GCMS, it is a source of contamination of the injection port liner and the GC column. Furthermore, it can sometimes affect the analysis results, shifting the retention times of pesticides in the same chromatographic region as caffeine. The method which employs a solid phase cartridge, as shown in Fig. 4, is time-consuming, and increases the number of analyses. For this study caffeine was removed using a simple procedure that exploits the physical properties of caffeine.

4.1 Caffeine Removal Study

Fig. 5 shows the structural formula of caffeine. The high solubility of caffeine in polar solvents permits large amounts of caffeine to be dissolved in the extraction solvent, e.g. acetone. Moreover, as the temperature of the solution rises, even greater amounts of caffeine are dissolved.

Japan's Positive List Test Method for GCMS analysis of pesticides specifies an extraction solvent having a 1:1 ratio of acetone and hexane. Here we considered the physical property of polarity, with acetone as a polar solvent, and hexane a non-polar solvent. Because the polar solvent accounts for half of the ratio of the solution, this solvent mixture is thought to permit caffeine to dissolve easily. Therefore, it was decided to use hexane alone as the solvent, eliminating the use of acetone.

In addition, a lower solution temperature was considered to provide the benefit of impeding the dissolution of caffeine, and therefore the sample extracts were stored in a freezer. This freezing of the solution is referred to below as "freeze processing."

When the sample solution was freeze processed, deposits became suspended in solution (Fig. 6). By applying centrifugation, these deposits were precipitated, and analysis of the supernatant was equivalent to analysis without most of the caffeine. This process was applied to a green tea sample for confirmation of the effect.

To confirm the effect on pesticide recovery using hexane as the extraction solvent along with the application of freeze processing, pesticides were added to a green tea sample solution that was previously subjected to the described pretreatment, and then we applied the change in solvent (using only hexane) in addition to freeze processing. No adverse effect on recovery was observed.

C8H10N4O2
Mol. Wt.:194.19

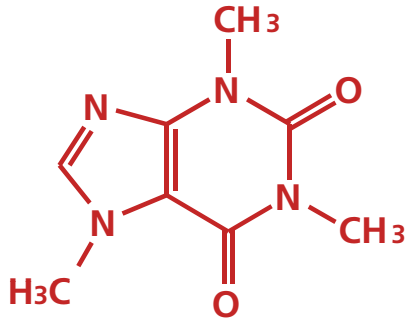


Fig. 5 Structure of Caffeine

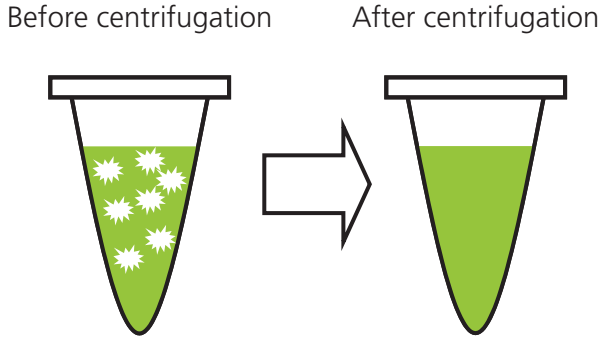


Fig. 6 Status at Vial Tip Before and After Centrifugation

4.2 Caffeine Removal Study Results

After preparing a caffeine-saturated hexane solution, the effect of freeze processing on caffeine removal was evaluated. The chromatograms of caffeine generated before and after processing are shown in Fig. 7, and the caffeine peak area ratio comparison is shown in Table 4. The caffeine content was reduced by 63.5 % as a result of freeze processing.

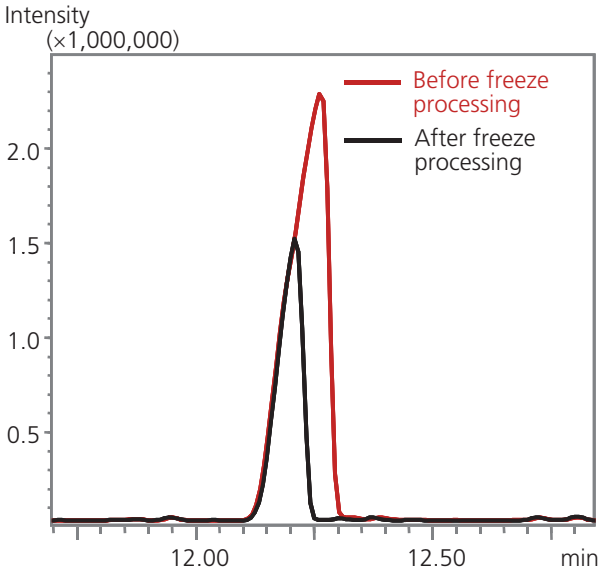


Fig. 7 Effect of Freeze Processing

Table 4 Comparison of Caffeine Area Ratios Before and After Freeze Processing

Before Freeze Processing	After Freeze Processing	Reduction Rate (%)
5209488	1906330	63.5

When freeze processing is conducted, deposits become suspended in the solution. Since centrifugal separation is known to effectively remove these suspended particles, centrifugation time was investigated to determine its effect on the separation. However, any rise in temperature that would occur during longer centrifugation would lessen the effect of freeze processing. Fig. 8 shows the results of the study of centrifugal separation time, allowing for precipitation of deposits following freeze processing. It took at least 1 minute to attain centrifugal separation, but as the centrifugal separation time increased beyond 1 minute, the caffeine area values increase accordingly, until they become constant after 3 minutes. Thus, at 3 minutes, it is thought that the effect of freeze processing would become counterproductive. From this result, we determined that the optimum time required for centrifugal separation is 1 minute.

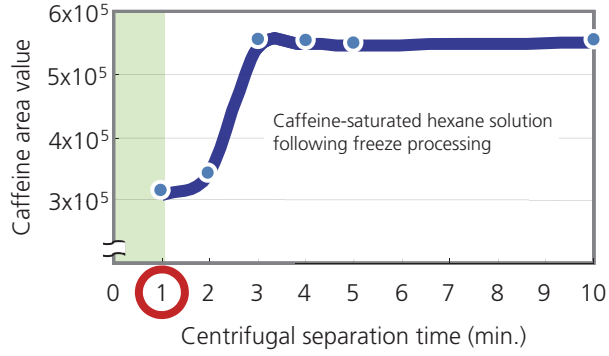


Fig. 8 Result of Investigation of Centrifugal Separation Time

4.3 Applying Caffeine Removal Operation to an Actual Sample

The caffeine removal operations described in the above study were employed to remove caffeine from a real-world tea sample. After processing commercially available tea according to Japan's Positive List Test Method, the solvent that had been specified for use in the obtained final solution was replaced with hexane, followed by freeze processing (-20 °C) and centrifugal separation (1 minute). The obtained supernatant was then analyzed by GCMS. Fig. 9 shows a flow chart of Japan's Positive List Test Method + caffeine removal operations.

Fig. 10 shows the TIC chromatograms obtained before and after caffeine removal by freeze processing. Prior to the removal processing, the caffeine peak is detected as a broad peak that exceeds the column load capacity, but after caffeine removal, a sharp caffeine peak within the column load range is seen, indicating that most of the caffeine was removed. As for the stability of the caffeine removal operation, the repeatability of caffeine area values following caffeine removal operations is shown in Table 5. Caffeine removal by freeze processing can thus be considered to be a stable process that provides good repeatability.

Caffeine removal by solvent replacement and freeze processing was thus confirmed, however actual target pesticides might be removed along with the caffeine. Therefore, spike and recovery testing was performed with respect to the above caffeine removal operation. A test solution prepared using Japan's Positive List Test Method was spiked with a standard mixture of pesticides, and after subjecting this solution to the above described caffeine removal processing, the pesticide recovery rates were obtained.

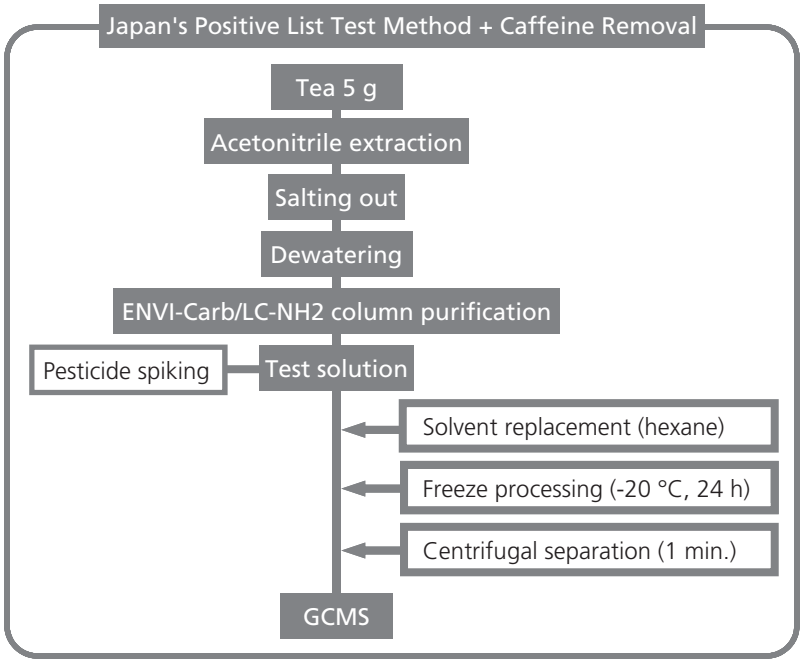


Fig. 9 Flow Diagram of Japan's Positive List Test Method + Caffeine Removal

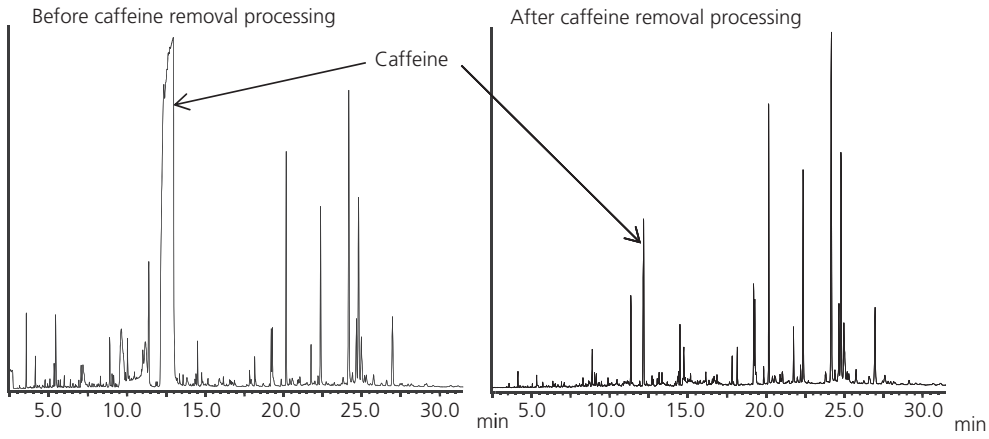


Fig. 10 Effect of Caffeine Removal by Freeze Processing

Table 5 Repeatability of Caffeine Removal Effect

	1	2	3	Average Value	CV%
Caffeine Area Values	4963206	5010227	4996268	4989900	0.48

Fig. 11 shows the pesticide spike and recovery test results obtained after caffeine removal processing. Recovery for most compounds fell between 80 and 120 %, and it was confirmed that switching to a hexane solvent and use of freeze processing did not result in significant loss of pesticides.

Not only was the caffeine peak drastically reduced as a result of the caffeine removal processing, other contaminant substances were eliminated. Removal of these contaminants lessened the adverse effects on the pesticide peak shapes. Fig. 12 shows examples of improved peak detection.

In the case of Mevinphos and Fosthiazate, the interfering peaks before these compounds were removed to allow clear detection of these pesticides. As for Propoxur, the *m/z* 152 peak shape was very different from the *m/z* 110 peak, but this improved as a result of freeze processing. Carbofuran, which co-eluted with another peak, became a single Carbofuran peak.

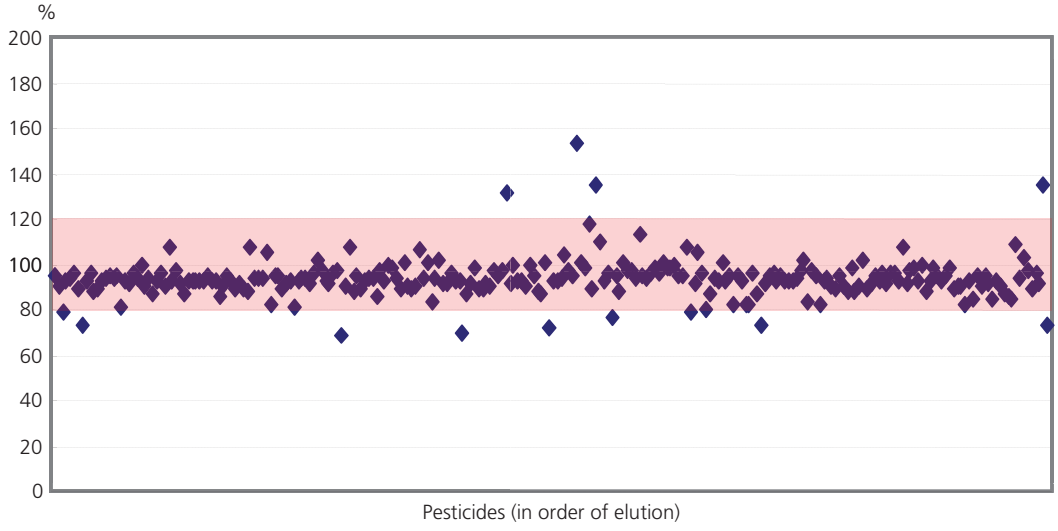


Fig. 11 Pesticide Spike and Recovery Test Results Due to Caffeine Removal Processing

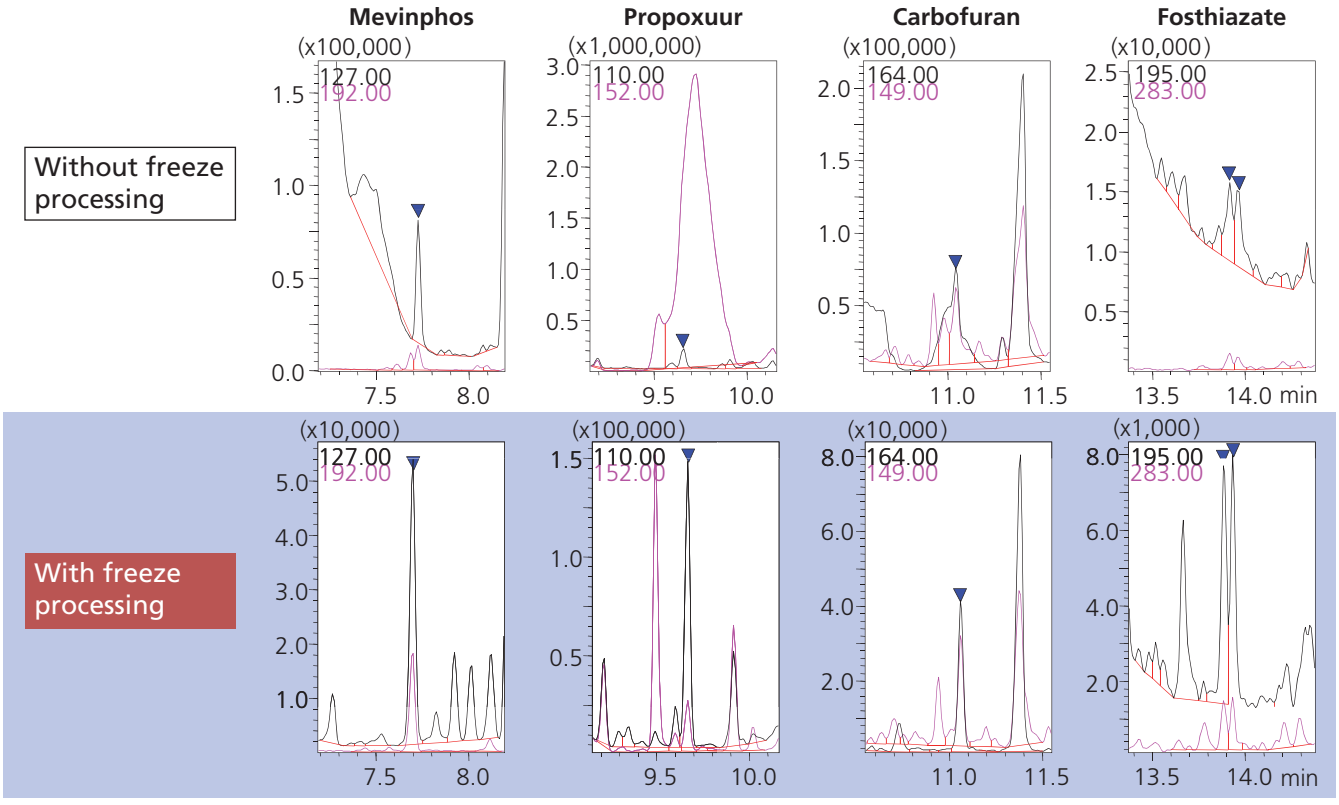


Fig. 12 Examples of Effect of Caffeine Removal Processing (green tea 0.1 ppm)

5. Conclusion

Pesticide residue analysis in tea using Japan's Positive List Test Method for GCMS simultaneous analysis yields good results, but during pesticide analysis by GCMS, the large amount of caffeine that remains in these tea samples can contaminate the GC injection port and column. Similarly, the presence of caffeine can co-elute with and mask the presence of pesticide residues in the same region of the chromatogram. To eliminate the interfering effects of caffeine, the solvent was changed from acetone : hexane (1:1) to 100 % hexane followed by freeze processing, exploiting the physical properties of caffeine, thereby efficiently decreasing the presence of caffeine in the sample. Further, utilizing this removal process, a method was developed for removing caffeine while retaining good recovery and minimal loss of the target pesticides.

*This document is based on information valid at the time of publication. It may be changed without notice.

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