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Non-target High Throughput Screening Method for 765 Multigroup and Multiclass Pesticides and Chemical Contaminants in Fruits and Vegetables by GC/LC-Q-TOF/MS

—AOAC Collaborative Study Implementation Protocol (First Draft)

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1.Purpose of the AOAC inter-collaborative study

It is very hard for the low-resolution mass spectrometry to monitor over 200 pesticides and other chemical contaminants in a single determination due to its limitations on dewell time and scanning speed, and the repeated determinations of samples are time-consuming and laoborious. Besides, reference samples are required for qualitative and quantitative determinations as comparison. These factors increase the time needed for detection and the relative costs, inhibiting the promotion of the pesticide multi-residue analytical technologies; In addiiton, the selective ion monitoring mode in low-resolution mass spectrometry adopted for multi-residue analysis is one targeted analytical technology, ignoring the potent non-target compounds and failing to fully meet the demand of improving the detection capability for food safety hazards. This collaborative study has adopted high-resolution mass spectromety, which makes true the replacement of conventional methods of using substantial standards as comparison with digital standards and a frog-leap development from target detection to non-target screening with the automation and informatization of the analytical technology at the same time. In this collaborative study, GC-Q-TOF/MS and LC-Q-TOF/MS are adopted to evaluate whether the sensitivity, repeatability, and reproducibility of the method for the 765 pesticide multigroup and multiclass residues in fruits and vegetables are capable of meeting the criteria for an AOAC offical method.

2. Application scope of the method

The method is appicable to GC-Q-TOF/MS and LC-Q-TOF/MS qualitative screening and quantitative analysis of 765 pesticides and chemical contaminants in 18 categories of 146 fruits and vegetables. Four kinds of fruit are chosen from them in particular: (1) apples (rengo); (2) grapefruit (citrus); (3) grapes (berry and other small fruits); (4) watermelon (melons); Vegetables: (1) spinach (leaf vegetables, green leaves); (2) celery (leaf vegetables, petioles); (3) cabbage (Amaranthus); (4) tomato (solanum). The method efficiencies are evaluated respectively regarding 494 pesticides by GC-Q-TOF/MS and 565 by LC-Q-TOF/MS.

For LC-Q-TOF/MS: Spiked recoveries and precision of 565 pesticides are investigated at respective concentrations of 5 μg/kg, 10 μg/kg and 20 μg/kg. Limit of screening of the method is investigated through matrix spiked experiments at concentrations of 1 μg/kg, 5 μg/kg, 10 μg/kg, 20 μg/kg, and 50 μg/kg. Besides apples (49.1%) and spinach (48.8%), the percentage of pesticides with a screening limit of 1 μg/kg in all other six substrates was greater than 50%. Pesticides with screening limit concentrations lower than 10 μg/kg account for 73.2%-81.9% of the total pesticides in the eight matrices.

For GC-Q-TOF/MS: SpikedSpiked recoveries and precision of 494 pesticides are investigated at respective concentrations of 10 μg/kg, 50 μg/kg, and 100 μg/kg. Limit of screening of the method is investigated through matrix spiked experiments at concentrations of 1 μg/kg, 5 μg/kg, 20 μg/kg, 50 μg/kg, and 100 μg/kg at the same time. In the eight matrices, majority of pesticides have the limit of screeing at 5 μg/kg, accounting for 40.0%-52.5% of the total pesticides; there are less than 20 pesticides with limit of screening at 100 μg/kg in these eight matrices, accounting for 4.0% of the total pesticides. There are 348-399 pesticides with limit of screening concentrations lower than 10 μg/kg in these eight matrices, accounting for 71.7%-82% of the total pesticides. (统计结果依赖 970 的结果如果改成 970)

3. The analytical procedures using GC-Q-TOF/MS and LC-Q-TOF/MS

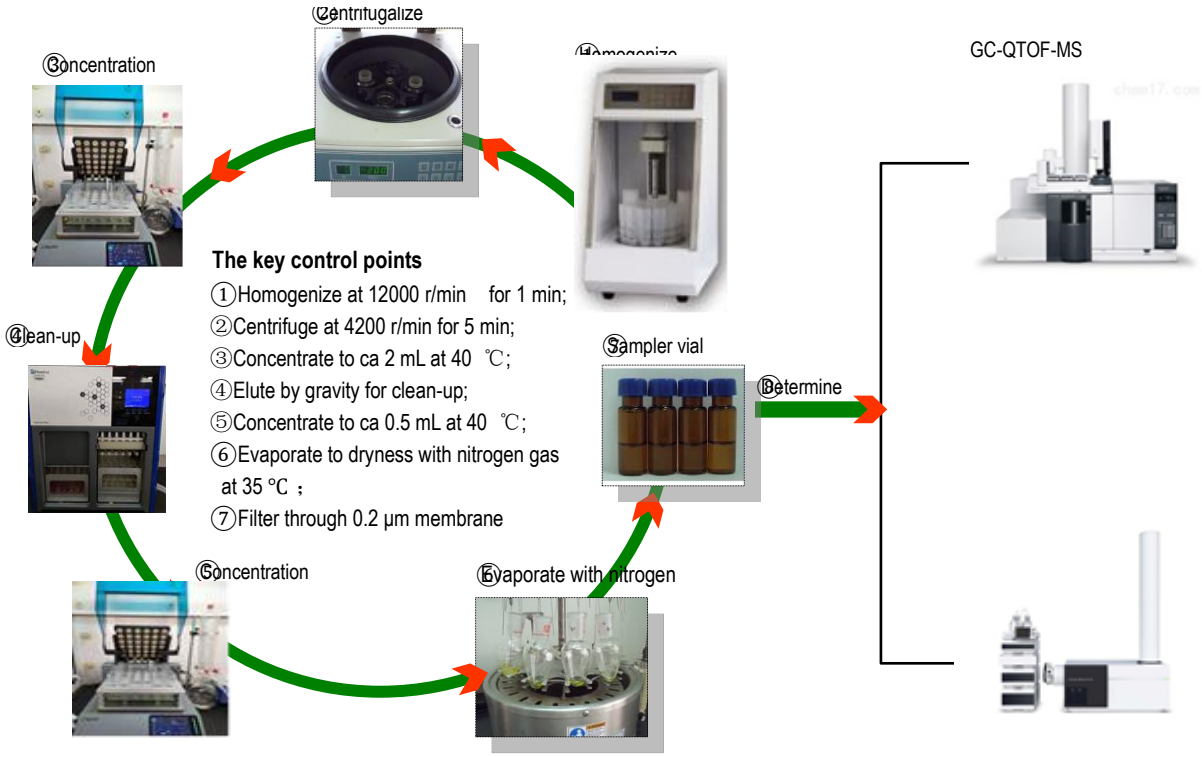

Fig.1. Analytical procedures

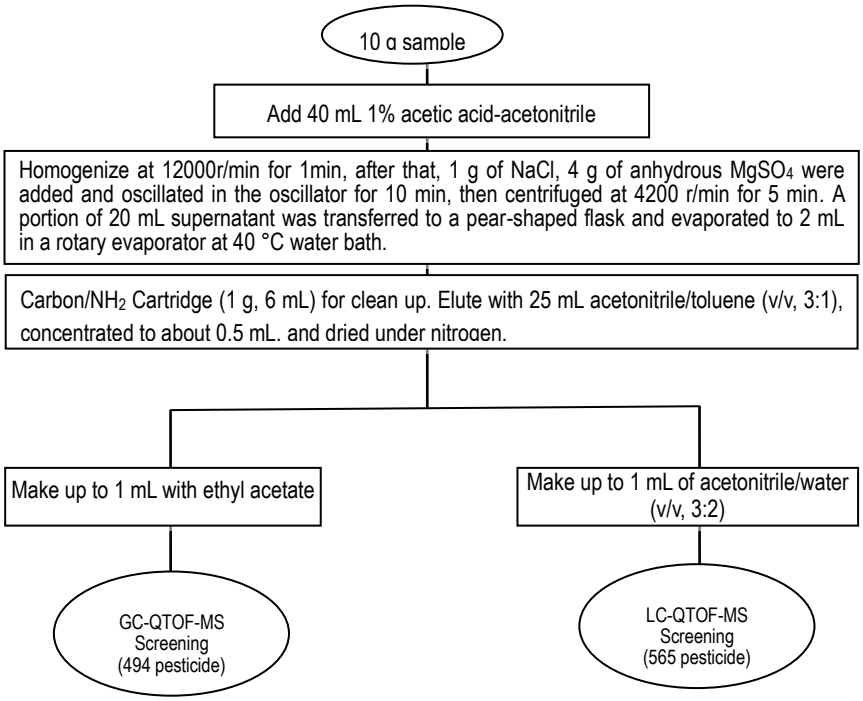

Fig.2 The analytical procedures using GC-Q-TOF/MS and LC-Q-TOF/MS

Study Director here solemnly reminds that the AOAC inter-collaborative study is a unified and standardized operation participated by the relavant laboratories of different countries. Each step of the experiment including the change of any testing reagent materals can only be adopted in the offical collaborative study after qualifications are obtained through practice and verifications in the pre-collaborative study stage and notes should be affixed in the corresponding columns in Table 1 of Annex B Excel worksheet, which must been complied by the volunteer participants

4.“Shrunken” AOAC inter-collaborative study protocol

If an inter-collaborative study is organized for determination of hundreds of pesticides, it will surely bring the participants with unimaginable difficulties in resources, time and personnels. Therefore, an “abridged” inter-collaborative study protocol has been proposed. It includes total four types of matrices: two fruits (apples and grapes) and two vegetables (tomatoes and cabbages). Two groups of pesticides (20 each) will be determined using GC-Q-TOF/MS and LC-Q-TOF/MS, respectively. Among the two groups of pesticides, there are 11 common pesticides and the total types of pesticides are 29. These 29 pesticides have been chosen from the 765 pesticides, as seen in Table 1-1 and Table 1-2 (11 common pesticides). They are representative among the total 765 pesticides detected by GC-Q-TOF/MS and LC-Q-TOF/MS, which belong respectively to organonitrogen, organichalogen, organophosphorus, organicsulphur, carbamate, and others; the catergories per function include: insecticides, herbicides, fungicide, as seen in Tables 2 and 3. These pesticides are very popular and widely applied in the fruits and vegetable, which are frequently required for detection in the international trade. In addition, these pesticides once applied in fruits and vegetables exhibit relatively good stability, and their physicochemical properities such as polarities are also widely representative.

Table 1 LC-Q-TOF/MS representative pesticide list

No.	Name	CAS No.	Apple	Grape	Cabbage	Tomato

			LOQ	Linear range	R^2	LOQ	Linear range	R^2	LOQ	Linear range	R^2	LOQ	Linear range	R^2
1	Acetochlor	34256-82-1	1	1~200	0.9875	5	5~200	0.9990	1	1~200	0.9908	1	1~200	0.9952
2	Ametryn	834-12-8	1	1~100	0.9893	1	1~200	0.9895	1	1~50	0.9913	1	1~50	0.9857
3	Benalaxyl	71626-11-4	1	1~100	0.9973	1	1~200	0.9987	10	10~200	0.9972	1	1~200	0.9972
4	Diphenamid	957-51-7	1	1~100	0.9965	1	1~200	0.9825	1	1~50	0.9873	1	1~200	0.9816
5	Esprocarb	85785-20-2	1	1~100	0.9976	1	1~200	0.9967	1	1~200	0.9923	1	1~200	0.9982
6	Fenothiocarb	62850-32-2	1	1~200	0.9895	1	1~200	0.9921	1	1~50	0.9931	1	1~200	0.9941
7	Mepanipyrim	110235-47-7	1	1~100	0.9965	1	1~200	0.9982	2	2~200	0.9931	2	2~200	0.9989
8	Metalaxyl	57837-19-1	1	1~200	0.9866	2	2~200	0.9818	1	1~50	0.9819	1	1~100	0.9813
9	Methoprotryne	841-06-5	1	1~200	0.9970	1	1~200	0.9974	1	1~200	0.9968	1	1~200	0.9996
10	Metolachlor	51218-45-2	1	1~200	0.9955	1	1~200	0.9967	1	1~200	0.9985	1	1~200	0.9999
11	Orbencarb	34622-58-7	1	1~100	0.9855	5	5~200	0.9989	2	2~200	0.9915	1	1~200	0.9979
12	Pentanochlor	2307-68-8	10	10~100	0.9938	2	2~200	0.9941	1	1~50	0.9955	2	2~200	0.9852
13	Picoxystrobin	117428-22-5	1	1~200	0.9902	1	1~200	0.9931	1	1~100	0.9878	1	1~200	0.9939
14	Pirimiphos-methyl	29232-93-7	1	1~100	0.9983	1	1~200	0.9985	1	1~200	0.9948	1	1~200	0.9968
15	Propisochlor	86763-47-5	10	10~200	0.9966	2	2~200	0.9952	2	2~200	0.9918	5	5~200	0.9992
16	Quinalphos	13593-03-8	1	1~200	0.9977	1	1~200	0.9852	1	1~100	0.9805	1	1~200	0.9993
17	Sebuthylazine	7286-69-3	1	1~200	0.9895	1	1~200	0.9914	1	1~50	0.9960	1	1~200	0.9930
18	Simeton	673-04-1	1	1~200	0.9805	5	5~200	0.9851	1	1~50	0.9904	5	5~100	0.9811
19	Terbuthylazine	5915-41-3	2	2~200	0.9870	1	1~200	0.9976	1	1~200	0.9963	1	1~200	0.9994
20	Tetraconazole	112281-77-3	1	1~100	0.9965	1	1~200	0.9975	r<0.99		0.9447	1	1~200	0.9997

（注明：0.9805 ,已经与陈辉确认过）

Table 1-2 GC-Q-TOF/MS representative pesticide list

No.	Name	CAS No.	Apple			Grape			Cabbage			Tomato		
			LOQ	Linear range	R^2	LOQ	LOQ	Linear range	R^2	LOQ	LOQ	Linear range	R^2	LOQ
1	Acetochlor	34256-82-1	5	5~200	0.9845	10	10~200	0.9969	5	5~200	0.9987	1	1~200	0.9906
2	Ametryn	834-12-8	1	1~200	0.9820	1	1~200	0.9957	1	1~200	0.9970	1	1~100	0.9962
3	Atrazine	1912-24-9	1	1~200	0.9825	2	2~200	0.9877	1	1~200	0.9933	1	1~100	0.9928
4	Benalaxyl	71626-11-4	1	1~200	0.9856	2	2~200	0.9984	2	2~200	0.9987	5	5~200	0.9992
5	Benzoylprop-ethyl	22212-55-1	2	2~200	0.9871	5	5~100	0.9977	1	1~100	0.9969	5	5~200	0.9900
6	Diphenamid	957-51-7	1	1~200	0.9942	2	2~200	0.9880	1	1~200	0.9955	2	2~200	0.9981
7	Isoprothiolane	50512-35-1	1	1~200	0.9843	1	1~200	0.9971	1	1~200	0.9993	1	1~200	0.9997
8	Kresoxim-methyl	143390-89-0	1	1~50	0.9951	2	2~50	0.9957	1	1~200	0.9862	2	2~200	0.9859
9	Methoprotryne	841-06-5	1	1~200	0.9812	1	1~200	0.9944	1	1~200	0.9955	1	1~100	0.9938
10	Metolachlor	51218-45-2	1	1~200	0.9953	2	2~200	0.9989	5	5~200	0.9979	5	5~200	0.9946
11	Orbencarb	34622-58-7	1	1~200	0.9861	1	1~200	0.9936	1	1~200	0.9964	2	2~100	0.9932
12	Penconazole	66246-88-6	1	1~200	0.9869	1	1~200	0.9968	1	1~200	0.9971	1	1~100	0.9926
13	Picoxystrobin	117428-22-5	1	1~100	0.9899	1	1~200	0.9933	2	2~200	0.9920	1	1~200	0.9814
14	Pirimicarb	23103-98-2	1	1~200	0.9957	1	1~200	0.9931	1	1~200	0.9965	1	1~50	0.9958
15	Quinalphos	13593-03-8	5	5~50	0.9972	2	2~50	0.9994	2	2~200	0.9850	1	1~100	0.9891
16	Simeconazole	149508-90-7	1	1~200	0.9933	2	2~200	0.9995	1	1~200	0.9984	2	2~200	0.9953
17	Tebufenpyrad	119168-77-3	1	1~200	0.9832	1	1~200	0.9950	1	1~200	0.9983	1	1~50	0.9970
18	Terbuthylazine	5915-41-3	1	1~200	0.9855	1	1~200	0.9968	1	1~200	0.9976	1	1~100	0.9959
19	Tetraconazole	112281-77-3	1	1~200	0.9899	1	1~200	0.9945	1	1~200	0.9967	1	1~100	0.9994
20	Thiazopyr	117718-60-2	1	1~200	0.9996	5	5~200	0.9989	10	10~200	0.9923	2	2~200	0.9994

Table 2 20 pesticides detected by GC-Q-TOF/MS

classified per chemical compositions	classified per functions

Carbamate	1	Herbicide	10
Organophosphorus	1	Insecticide	3
Organohalogen	4	fungicide	7
Organic sulfu	1		
Organonitrogen	11		
Other	2		

Table 3 20 pesticides detected by LC-Q-TOF/MS

classified per chemical compositions		classified per functions	
Carbamate	2	Herbicide	12
Organophosphorus	2	Insecticide	3
Organonitrogen	12	fungicide	5
Organohalogen	3		
Other	1		

5. Sample quantity and residue concentration levels of target pesticides in the collaborative study

Each participating laboratory makes a total determinaton of 24 collaborative study samples (16 spiked samples and 8 aged samples). Each sample is only enough for one single experiment, namely, each participating lab has only one chance for each sample and must ensure absolute success without any errors. Therefore, Study Director hereby reiterates that this collaborative study will have to deal with utomst care and conscientiousness. These 24 official collaborative study samples are illustrated in Fig.3.

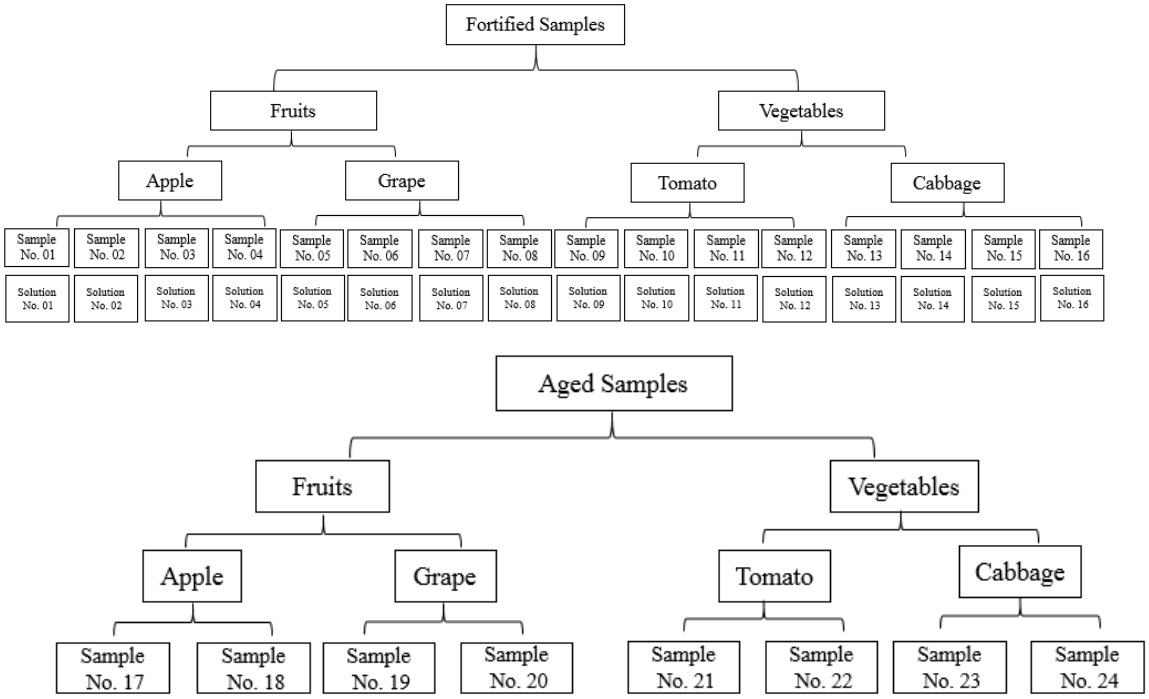

Fig.3. Number of Spiked and Pesticide Aged Samples for the Collaborative study

The serial numbers here only represent the design thoughts of the collaborative study protocol instead of the actual sample number to be shipped to the participant later. For spiked samples, each sample is spiked at two concentration levels, low and high, and each level is duplicate. For the aging samples, the types of pesticides contained in each matrix are different and the concentrations are different, and one of the samples is a blank.

6. AOAC collaborative study process

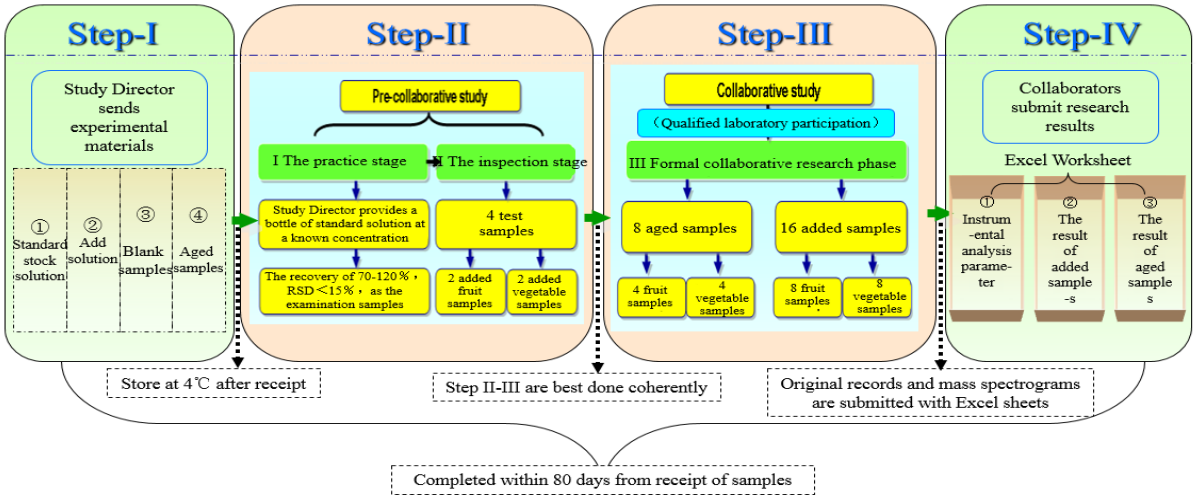

Fig. 4. The flow chart of the AOAC collaborative study

7. Testing materials sent to the participating lab by Study Director

Study Director will supply two packages of testing materials used for the collaborative study by GC-Q-TOF/MS and LC-QTOF/MS, respectively. The collaborative study lab will receive the corresponding testing material packages depending on which method the lab plans to apply for the study, and the reception sheet of the collaborative study lab is shown in Annexs Table1-1 and Table 1-2.

Each test material package will contain the items as follows:

7.1 Quality control standard stock solutions

2 ampoules (1.0 mL ×2, including 1 spare), which is used for checking the sensitivity and stability of the instrument. (Detailed information such as the name and concentration of specific pesticides will be provided when the collaborative study is formally carried out)

7.2 For Pre-collaborative study material

1) 20 pesticide mixed standard stock solutions: 2 ampoules (1.0 mL ×2，including 1 spare).
2) Internal standard stock solutions; 2 ampoules (1.0 mL ×2，including 1 spare).
3) Fruit and vegetable blank samples: 1 bottle each for apples, grapes, tomatoes and cabbages.
4) Examination samples:
   ①Spiked samples: 4 ampoules, used for preparaton of examination samples;
   ②Fruit blank samples: a portion for apples and grapes respectively, used for preparation of test samples.
   ③Vegetable blank samples: a portion for tomatoes and cabbages respectively, used for preparation of test samples.

7.3 For Collaborative study materals

1) 20 pesticide mixed standard stock solutions: 2 ampoules (1.0 mL ×2，including 1 spare), used for establishing at least 5-point matrix-matched internal standard calibration curve.
2) Internal standard stock solutions: 2 ampoules (1.0 mL ×2，including 1 spare).
3) Spiked solutions: 16 ampoules, used for preparaton of spiked samples.
4) Fruit and vegetable blank samples: 16 portions, No.01-No.04 standing for apples, No.05-No.08 grapes, No.09-No.12 tomatoes, No.13-No.16 cabbages, used for preparation of spiked

samples.

5) Fruits and vegetable aged samples: 6 portions, No.17 and No.18 standing for apples, No.19 and No.20 grapes, No.21 and No.22 tomatoes and No.23-No.24 cabbages.
6) Apples, grapes, tomatoes and cabbages blank samples: 1 ampoule each, used for preparation of matrix-matched blank sample solutions

For laboratories that cannot buy Carbon/NH_2 cartridges, Study Director can provide the required Carbon/NH_2 cartridges.

8. AOAC Pre-collaborative study

8.1 Significance of the pre-collaborative study

Although fruit and vegetable matrices are relatively simple, this collaborative study involves comparatively high technical skills and is of certain difficulty compared with the low-resolution mass spectrometry such as LC-MS/MS or GC-MS/MS because it is the first inter-collaborative study that adopts GC/LC-Q-TOFMS for the screening of pesticides in fruits and vegetables, so it is abosolutely necessary for each collaborator to conduct the pre-collaborative study. The purpose of the pre-collaborative study is just like the excellent explanations made by Ms. Krystyna Mclver, AOAC Sr. Director of Stakeholder Communications, ""pre-collaborative study by the participating laboratories to ensure the laboratories are qualified to run the method".

Study Director reminds each collaborator again: for each of the 24 collaborative study samples, each collaborator has only one chance for experiment no matter wether it is a success or failure. Study Director, however, has provided each collaborator with practice samples sufficient for his or her skillful mastery of key control points of such technology through adequate practice, hoping that he or she will cherish the practice opportunitiees provided by this collaborative study.

8.2 Pre-collaborative study acceptance criteria

1) For GC-QTOF/MS or LC-QTOF/MS, at least provide 5-point matrix-matched internal standard calibration curve, with the linear correlation coeffient of $R^2 \geq 0.98$.
2) Recoveries within the range of 70%-120%, and RSD＜20％(n=5).
3) Confirmation of the pesticide to be detected shall meet the requirement regulated in the EU guideline of SANTE/11813/2017) [2].
    a) Retention time deviations ±0.2 min;
    b) At least two ions.For LC-QTOF/MS, one of them should be preferably molecular ion, and the other be secondary fragment ion; and for GC-QTOFMS, there should be 2 fragment ions. Mass deviation less than 5ppm for molecular ion and 10 ppm for fragment ion; for those less than 200 Da, mass deviation may be within ±1 mDa.
    c) S/N≥3.
    d) For GC-QTOF/MS，ion ratio within ±30% of average of calibration standards from the same sequence.
    e) For pesticides detected by qualitative screening, none of the collaborators shall be permitted to turn out false positive results, but allowing for the existence of 5% false negative results.

Study Director renders a special reminder: the success of the collaborative study will mainly depend on whether the examination results from the pre-collaborative study of the

collaborators will be able to meet the above-mentioned acceptance criteria. In terms of pre-collaborative study, any disqualifications from qualitative precision, recoveries, RSD or R^2 exceeds 30% of the total number, the official collaborative study sample will be discontinued in principle.

8.3 Practice content of the pre-collaborative study

8.3.1 Preparation of three standard working solutions (pesticide mixed standard working solutions, internal standard woking solutions and quality control standard working solutions)

Mixed standard stock solutions, internal standard stock solutions and quality control standard stock solutions related to pesticides for use in the pre-collaborative study are to be supplied by Study Director, and their volume is 1.0 mL, which should be used after being diluted into standard working solutions. The detailed steps are as follows: any ampoule bottle containing stock solutions will be erected till all the solutions at the top of the ampoule bottle flow down the inner walls before opening the bottle with care and transferring the solutions into a 10 mL volumetric bottle. To ensure the total transfer, 3×1mL methanol is used to cleanse the bottle, while the cleansing liquid is transferred together into the 10 ml volumetric bottle before being diluted to graduation level with methanol, and the corresponding standard working solutoins are made after homogenization. The concentrations of standard working solutions are shown in the collaborative study methods of **Section** 5.11 and Tables 1 and 2. The standard working solutions shall kept from exposure to any light and in the referigerator at 4 ℃ for spare use.

8.3.2 Establishing GC-QTOF/MS analytical process

1) According to the chromatographic conditions stated in the collaborative study method (a)-(f) of **Section** 6.3.1, select the chromatographic column and setup the instrument paramerters, to establish the GC analytical method. Setup the GC-QTOF/MS mass spectrometry acquisition conditions as stated in the collaborative study of (g)-(m) in **Section** 6.3.1.
2) Updating retention time for GC-QTOF/MS: pipette 10 μL of pesticide mixed standard working solutions and 10 μL of the internal standard prepared in **Section** 8.3.1 into a 2 mL injection vial, which is further dried with nitrogen gas and diluted with 1 mL ethyl acetate. Calculate the average retention time (RT) of each pesticide acquired from 5 continuous injections according to the data acquisition method (n=5), and then update the RT in the PCDL database.

8.3.3 Establishing LC-QTOF/MS analytical process

1) According to the chromatographic conditions of **Section** 6.3.2 (a)-(d) of the collaborative study method, select the chromatographic column and setup the chromatographic parameters such as the mobile phase elution gradient program (see Collaborative Study Method Table 3). According to the mass spectrometry parameters of **Section** 6.3.2(e)-(n) of the collaborative research method, setup the mass spectrometry acquisition method such as the ion spray voltage, nebulizer gas pressure, and collision energy of each pesticide (see Table 4 of the collaborative research method).
2) Updating the retention time of each pesticide for LC-QTOF/MS: pipette 50 μL of pesticide mixed standard working solutions and 40 μL of the internal standard working solutions prepared in **Section** 8.3.1 into 2-mL injection vial, which is then dried by nitrogen gas and diluted using 1 mL of acetonitrile:water (v/v=3:2). Calculate the average retention time from 5

continuous injections using the same acquisition method beofore updating RT in the PCDL databases.

Study Director reminds every collaborator: the analytical parameters presented in the method are obtained from the instrument of Agilent for demonstrations. The preliminary results of analytical parameters such as those from other instrument vendors like Waters and Applied Biosystems are tablulated in Annex A for references by the collaborators. Only after the collaborators have met the acceptance criteria with their practice test results through the AOAC pre-collaborative study and spiked experiment confirmation and practice examinations regarding fruits and vegetables, can the analytical parameters from these instruments be adopted in the official collaborative study, and the analytical parameters from the instruments will be required to be filled in Table 1 of Annex B worksheet of the collaborative study method.

8.3.4 Using quality control standard working solutions to check the sensitivity and stability of the instrument

Prior to determination, quality axis calibration should be conducted for GC-QTOF/MS and/or LC-Q-TOF/MS per requirements, and the sensitivity and stability of the instrument should be checked after the requirements are met.

Before making determination of each lot of samples, a successive injection of 1.0 μL (GC-QTOF/MS) or 5.0 μL (LC-QTOF/MS)quality control standard working solutions will be conducted (see 8.3.1), and if the deviation in retention time of pesticide peaks over two successive injections does not exceed 0.1 min (GC-Q-TOF/MS) or 3% (LC-Q-TOF/MS) of each time and the deviation in the response values is not 6% greater than each time while S/N ratio of the internal standard higher than 1000 for GC-QTOF/MS or higher than 500 for LC-QTOF/MS, the sensitivity and stability of the instrument is deemed to comply with the requirements, which is fit for sample determinations. Otherwise, maintenance measures such as replacing the inner linings of injection inlet, insulators, chromatographic columns and cleansing of ions sources are needed to be taken for GC-QTOF/MS; maintenance measures such as replacing chromatographic columns and cleansing of ion sources are required to be taken for LC-QTOF/MS.

8.3.5 Establishing 5-point matrix-matched internal standard calibration curve

For this collaborative study, matrix-matched internal standard calibration curve is adopted for quantification, and heptachlor epoxide (B) is used as internal standard for GC-QTOF/MS and atrazine-D5 as internal standard for LC-QTOF/MS. The process of establishing the 5 point matrix-matched internal standard calibration curve is as follows:

Prepare 5 portions of blank sample solutions for each type of the fruits and vegetables in accordance with the operational procedures of 6.1, 6.2.1 and 6.2.2 in the collaborative study method, add respectively 0.5, 2.5, 5, 25 and 50 μL pesticide mixed standard working solutions (see concentrations 10 ppm in 8.3.1) and add 20 μL heptachlor epoxide (B) internal standard working solutions (see concentrations 10 ppm in 8.3.1) for GC-QTOF/MS; add 20 μL atrazine-D5 internal standard working solutions (see concentrations 10 ppm in 8.3.1) for LC-QTOF/MS. Use nitrogen gas to dry each calibration solution in a water bath at 35 ℃, dissolve the residues with 1.0 mL of ethyl acetate for GC-QTOF/MS or with 1 mL of acetonitrile/water (3:2, v/v) for LC-Q-TOF/MS, then homogenize with ultrasonication, pass through 0.20 μm filtering membrane.

It then results in the 5 point matrix-matched internal standard mixed solutions, which is used for establishing the 5 point matrix-matched internal standard calibration curve. The matrix-matched internal standard mixed solutions shoud be prepared right before it is used.

8.3.6 The practice of the collaborators on their own

Study Director reiterates that the official collaborative study is only enough for one analysis, while the samples supplied to you for practice on your own are plenty, which is sufficient for you to master the key points of the method through practice. It is hoped that you will make full use of the opportunity to get well acquainted with the method so as to ensure the success with the official collaborative study samples through a single experiment. Study Director hereby gives out a special reminder that the test samples can be detected only after your practice has met the acceptance criteria of the pre-collaborative study.

You may conduct spiked recovery experiments on your own at spiking levels of 10 μg/kg (10 g fruit and vegetable spiked with 5 μL of pesticide mixed standard working solutions) and 100 μg/kg (10 g fruit and vegetable spiked with 50 μL of pesticide mixed standard working solutions) by using fruit and vegetable blank samples and the prepared pesticide mixed standard working solutions supplied by Study Director (see 8.3.1). When your practice has met the acceptance criteria of the pre-collaborative study, you may undertake the test samples.

8.4 The examination content of the pre-collaborative study

8.4.1 Preparation and detection of fruit spiked samples: defrost and homogenize No.p1 and No.p2 capped centrifuge tubes containing apple and grape blank samples shipped by Study Director. Erect these two spiked solution ampoule bottles in front of the centrifuge tubes with identical serial numbers, wait till the top solutions in the bottle flow down the inner walls before opening the bottle and adding all the spiked solutions into the two centrifugal tubes with identical serial numbers, use 0.5 mL methanol to cleanse the ampoule bottle 3 times (do not use too much methanol to cleanse the bottle , or there will be plenty of pigments of co-extracts during sample extraction, affecting cleanup and detection) and transfer the cleansing liquid into the corresponding centrifugal tubes. Sit still for 20 min until the testing solutions are absorbed by samples before conducting sample preparations per the extraction in 6.1 and cleanup in 6.2 of AOAC collaborative study method. In the meanwhile, prepare 5 point matrix-matching internal standard calibration mixed solutions and establish the matrix-matching internal standard for quantitative determination of spiked samples of apples and grapes.

8.4.2 Preparation and determinaiton of vegetable spiked samples: the serial numbers of tomatoes and cabbages are No.p3 and No.p4 respectively, and add these two ampoules into the two centrifugal tubes with corresponding serial numbers per the method in 8.4.1. At the same time, prepare the 5 point matrix-matched internal standard calibration mixed solutions, and establish matrix-matched internal standard calibration curve for quantification of tomatoes and cabbages. (Pesticide varieties and concentrations added into apples, grapes, tomatoes and cabbages samples are different, but the pesticides contained in these four matrices all come from the Pesticide List in Table 1.

8.4.3 Auditing the analytical results in the following three aspects: (1) whether retention time within the required time window; (2) whether the choice of integration line correct; (3) whether the qualitative determination of pesticides complies with EU or AOAC standard.

8.4.4 The collaborator is to submit the analyical results of the pre-collaborative study test samples to Study Director.

The collaborators will fill out the analytical parameters in Table 1 of Annex B Excel worksheet in the collaborative study method; those who participate in GC-QTOF/MS will fill out the analytical results in Table 2 of Annex B Excel worksheet in the collaborative study; those who participate in LC-QTOF/MS will fill out the analytical results in Table 3 of Annex B Excel Worksheet in the collaborative study method. Tables 1-3 of Excel worksheet, mass spectra and raw data will be transmitted to Study Director as soon as possible.

8.5 Study Direcgtor will give the collaborators a feedback of the pre-collaborative study results

Study Director will give a reply to the collaborators on the test results within 48 hours in order for them to consider whether to continue the collaborative samples upon reception of the above-mentioned experiment result tables, mass spectra and raw data.

9. Official AOAC collaborative study

9.1 Preparing three standard working solutions (pesticide mixed standard working solutions, internal standard working solutions and quality control standard working solutions)

Pesticide mixed standard stock solutions, internal standard stock solutions and quality control standard stock solutions used for collaborative study are all supplied by Study Director, with volume 1.0 mL, which need to be diluted to standard working solutions for use. The detailed steps are identical with those in the practice stage. The concentrations of standard working solutions are shown in **Section** 5.11 and Tables 1 and 2 in the collaborative study method. Standard working solutions should be kept from any exposue to light and stored in referigerator at 4 ℃.

9.2 Establishin at least 5 matix matching internal standard calibration curve

The process of esblishing the 5 point matrix-matched internal standard calibration curve is the same as those in the practice stage. The linear correlation coefficient R^2 of each pesticide matrix-matched internal standard calibration curve should be greater than 0.98. In the actual determinations, each fruit and vegetable must be quanatified with the corresponding fruit and vegetable matrix-matched internal standard calibration curve, which are not to be confused. The matrix-matched internal standard calibration mixed solutions should be made for immediate use.

9.3 Preparation and determination of collaborative study spiked samples (4 spiked samples of apple are taken for demonstration).

Defrost and homogenize No.01,No.02, No.03 and No.04 capped centrifuge tubes containing apple blank samples supplied by Study Director. Erect these four spiked solution ampoule bottles in front of the centrifuge tubes with identical serial numbers, wait till the top solutions in the bottle flow down the inner walls before opening the bottle and adding all the spiked solutions into the 4 centrifugal tubes with corresponding serial numbers, use 0.5 mL methanol to cleanse the ampoule bottle 3 times (do not use too much methanol to cleanse the bottle , or there will be plenty of pigments of co-extracts during sample extraction, affecting cleanup and detection) and transfer the cleansing liquid into the corresponding centrifugal tubes, as seen in Table 5. Sit it still for 20 min until the testing solutions are absorbed by samples before having become the collaborative spiked samples and conducting sample preparations per the extraction in 6.1 and cleanup in 6.2

of AOAC collaborative study method.

Table 5. The Corresponding Serial Numbers of Blank Samples and Spiked Solution Ampoule Bottles

Serial Nos.	No.01	No.02	No.03	No.04
Blanks samples	No.01	No.02	No.03	No.04
Spiked solutions	No.01	No.02	No.03	No.04

Study Director gives a special reminder: (1) a portion of foritified solution corresponds with a portion of blank sample, which is only enough for a single analysis. The spiked solution concentration in each ampoule bottle is unknown to the collaborator, so one will have to be careful with the experiment and ensure a thorough transfer. (2) for the purpose of preventing the cross-interference of samples (especially high concentrations vs. low concentrations or vs. blank samples), one will have to conduct sample preparation and determination in accorance with the sequence of the serial numbers and give a down-to-earth cleansing of the sample preparation apparatuses after use each time so as to avoid contaminating the next sample preparation.

9.4 Sample preparation and determination of collaborative aged samples (2 aged samples of apples are taken for demonstration).

Sample preparation is conducted for aged samples of apple No.17 and No.18 according to 6.1 Extraction and 6.2 Cleanup in the collaborative study method. At the same time, prepare 5-point apple matrix-matched internal standard calibration mixed solutions and establish the matrix-matched internal standard calibration curve for quantification of aged samples of apple (see 8.3.5).

9.5 Analytical requirements for GC-QTOF/MS or LC-QTOF/MS

The past within-lab study discovers that there are auto integration errors about 5% for GC-QTOF/MS and 10% for LC-QTOF/MS, which needs manul correction. The frequency of such integration errors is closely related with the extent of contamination of the instrument.

Therefore, the following key control points should be given special attention to for the purpose of ensuring accuracy of qualitative and quantitative results.

Before determining the collaborative samples, use quality control standard working solutions to check if the sensitivity and stability meets the requirement.

After finishing determination of a batch of samples, use quality control standard working solutions to check the sensitivity and stability of the instrument to see whether they agree with each other before and after determination.

Strictly following the qualitative and quantitative requirements in the method, verify retention time and accurate mass fragments of each pesticide peak to ensure that the peaks of each target pesticide are within the integration window and correctly identified.

Verify if the choice of each pesticide peak integration line is correct, and for the pesticides with incorrect integration lines, manual integration mode from valley to valley should be adopted.

9.6 The collaborator submits official collaborative study results to Study Director

The collaborator will fill out the instrumental analytical parameters in Table 1 of Annex B Excel worksheet in the method after verification per requirements; he or she will fill out R^2, slope, intercept values of matrix-matched internal standard calibration curve used for quantification of collaborative samples and the content of target pesticides in Tables 4 and 5 of Annex B Excel worksheet in the method.

After completion of the above-mentioned work, the collaborator is kindly advised to transmit

the filled Tables 1, 4 and 5 of Excel worksheet together with mass spectra and raw data to Study Director. The related spetra includes;

1) Total Ion chromatogram of the reagent Blank samples
2) A set of matrix-matched internal standard curve used for quantification fruit and vegetable collaborative samples (including 20 pesticides).
3) A set of mass spectra with secondary accurate mass fragments for fruit and vegetable spiked samples (containg 20 pesticides and 1 internal standard).

Study director warns: special precaution should bet rendered that every step of the operational procedures be strictly followed. In case of any deviations, one is advised to put a note in the column appropriate in Tables 1-5 of Annex B Excel worksheet in the method. At the same time, comment or suggestions for the method are also welcome to be filled in the relevant columnof the above-mentioned tables.

10. Statistical analysis and judgment criteria of the collaborative results

(1) matrix-matched internal standard curve R2＞0.98.

(2) Outliers of experimental data shall be judged and eliminated through Dixon testing, and statistical analysis is conducted on the analytical results of spiked samples and aged samples. EU SANTE guideline [2] is adopted for identification of target pesticides in fruits and vegetables for the study. The judgment criteria of method recoveries, reproducibility and repeatability are based on AOAC standard, and see Table 6.

Table 6. Criteria for recoveries, RSD_r and RSD_R

Concentration	Recovery limits	Repeatability (RSD_r)	Reproducibility(RSD_R)
10 μg/g(ppm)	80-115%	6%	11%
1μg/g	75-120%	8%	16%
10 μg/kg(ppb)	70-125%	15%	32%

Study Director reconciles the analytical results from each collaborator and have them summarized in Table 7 (demonstration).

Table 7. Statistically analysis of data from collaborative study results (demonstration only)

Pesticide: Acetochlor									
Lab	Apple		Difference	SUM	Lab	Apple		Difference	SUM
Sample No.	S01-GC	S02-GC			Sample No.	S03-GC	S04-GC		
1					1				
2					2				
3					3				
…					…				
P					P				
Average,μg/kg					Average,μg/kg				
Add content, μg/kg					Add content, μg/kg				
The average recovery,%					The average recovery,%				
SR					SR				
RSDR,%					RSDR,%				
R					R				
Sr					Sr				
RSDr,%					RSDr,%				
r					r				

11. Requirement for adaptability of the instrument

Prior to determination, the mass calibration should be conducted for GC-QTOF/MS and/or LC-Q-TOF/MS per requirements, and the sensitivity and stability of the instrument should be checked after the requirements are met.

Before making determination of each batch of samples, a successive injection of 1.0 μL (GC-QTOF/MS) or 5.0 μL（LC-QTOF/MS）quality control standard working solutions will be conducted (see 8.3.1), and if the deviation in retention time of pesticide peaks over two successive injections does not exceed 0.1 min (GC-Q-TOF/MS) or 3% (LC-Q-TOF/MS) of each time and the deviation of the response value is not 6% greater than each time while S/N ratio of the internal standard higher than 1000 (GC-QTOF/MS) or higher than 500 (LC-QTOF/MS), the sensitivity and stability of the instrument is deemed to comply with the requirements, which is fit for sample determinations. Otherwise, maintenance measures such as replacing the inner linings of injection inlet, insulators, chromatographic columns and cleansing of ions sources are needed to be taken for GC-QTOF/MS; maintenance measures such as replacing chromatographic columns and cleansing of ion sources are required to be taken for LC-QTOF/MS.

Study Director: it is a must to check the consistency of the stability of the instrument before and after the analysis of each sample so as to avoid relatively big errors due to the lowering of sensitivity and stability turbulence from contamination of the instrument, etc.

For both GC-QTOF/MS and LC-QTOF/MS, analytical data processing software must be equipped.

12. SPE mass conditions

Based on numerous experiments done by our team, Carbon/NH_2 cartridge or equivalent is recommended for use in the study, but they should be equipped with Visiprep 5-port flask vacuum manifold (RS-SUPELCO 57101-U, Sigma Aldrich Trading Co., Ltd) or equivalent solid phase extraction equipment.

Fig.6 is the SPE equipment used in our lab for the reference by the collaborator.

Fig.6 SPE equipment

Visiprep 5-Port flask vacuum manifold from Supelco recommended here is because it is handy for receiving large volume of rinsing liquid . If the collaborator is able to solve the reception of big volume in the cleanup process, he or she may use other SPE devices.

13. Checking the interference and influence from test reagents

To prevent the interference from reagents, blank experiment should be conducted involving

all the reagents to confirm the absence of interfering peaks, and the collaborator is kindly advised to supply blank chromatograams to the Study Director.

14. Reventing cross-interference between the samples

To prevent the cross-interferce, the blade of homogenizer shall be thoroughly cleansed for 30 sec in the same mode as sample extraction with 2×50 mL acetonitrile after extraction of previous sample and before extraction of next sample in order to avoid the interference of samples with high pesticide content on those with low content or absence of pesticides, which is utmostly important in the collaborative study.

15. Analytical sequence requirements for the samples

For testing of the collaborative samples, Study Director suggests that it should be conducted per two groups.

First group: Before analyzing samples, use quality control standard working solutions to do a 2-3 times repeated sample injection (checking the instrumental sensitivity and stability), sample screening and repeatedly checking quality congtrol standard working solution 2-3 times (rechecking the instrumental sensitivity and stability).

Second group: Use quality control standard working solutions to do a 2-3 times repeated injection (checking instrumental sensitivity and stability), sample testing solution determination, conduct a successive injection of the matrix-matched standard solutions with the concentrations from low to high at points 1, 2, 3, 4 and 5, and finally do a repeated checking of quality control standard working solutions 2-3 times (rechecking instrument sensitivity and stability).

16. Requirements for the proficiency level of each collaborator

Each of the 24 collaborative samples serves as a blind sample for a single analysis. To attain an ideal analytical result, each collaborator must participate in the pre-collaborative study before analyzing the 24 collaborative samples. When his or her practice proficiency reaches the technical requirement, he may undertake the test samples and is advised to email the results to Study Director as soon as possible, who will reply to the collaborator with the judgment results of the test samples within 48 hours.

Study Director renders a special reminder: For test samples , any disqualifications from recoveries, RSD, ion abundance ratio or R^2 exceeds 30% of the total number, the official collaborative study sample will be discontinued in principle.

To achieve it, the collaborator should be equipped with following testing skills and experience:

1) Be proficient in sample prepration for pesticide residue analysis
2) Be proficient in operation of GC-QTOF/MS and LC-QTOF/MS with technique expertise (such as skillful operation, troubleshooting, and routine maintenance)
3) Have expertise in MS data elucidation technique

17. Deadline and requirements for completion of the collaborative study

When the AOAC Pesticide Chemical Contaminants Committee approves our protocol, we will spend two months to recruit collaborative research participants around the world, and strive to have 20-30 laboratories in 15 countries and regions to participate the collaborative studies.

After confirmation of the participating collaborators, we will immediately set about preparing the standard solutions, spiked solutions and the relevant testing consumables used for the

pre-study and official study, and ship them to the collaborator in a lumpsum via express delivery. The collaborator is required to finish the study within 80 days from the date of shipment of these testing materials from China.

At the same time, the collaborator is also encouraged to fill out the comments and suggestions in the column concerned in addition to the timely transmission of the tables of test results after completion of the collaborative samples.

The collaborator shall check at once if the testing material received agree with those in packing list and the materials are in sound condition and fill out the material reception form to be emailed or faxed to the Study Director upon reception of the collaborative materials. He or she is required to complete all the analytical tasks of testing samples within 80 days from the date of receiving the testing materials.

18. Safety precautions

Acetonitrile, toulene and other organic reagents used in the experiment pose certain harm or hazard to human bodies. Thereofore, a special reminder is given that emulsified gloves be worn in the experiment and operation be conducted with care in a well-ventilated enviornment.

19. Hope, requirements and committments

Study Director hopes that there are 20-30 laboratories participating in the collaborative study for each method (GC-QTOF/MS or LC-QTOF/MS) from 15 countries and regions.

Study Director requires each collaborator to finish the collaborative study within 80 days upon reception of the testing materials and transmit data sheet, spectra and raw data as well as his or her comments or suggestions for the method to the Study Director.

Study Director promises to give a reply to the collaborator regarding any technical questions raised by the collaborator during the study period with 48 hours; he will do his best to help the collaborator with other problems if there are any.

20. Contact

During AOAC collaborative study period, the collaborator may get in touch with Study Director any time by the following address:

Email: panggf@caiq.org.cn

21. Reference

(1) http://www.flworkshop.com/Community/pesticides.html
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(3) AOAC Guidelines for single laboratory validation of chemical methods for dietary supplements and botanicals
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22. Acknolwdgements