TERMS OF REFERENCE FOR R&D PROJECT

BUREAU OF INDIAN STANDARDS

Pesticide Residues Analysis Sectional Committee, FAD 27

1. Title of the Project

Development of multiresidue method for pesticide residues by LC-MS/MS and GC-MS/MS in edible oils.

2. Background

2.1 Edible oils are an important component of food products for daily life. Modern agricultural practices use a variety of pesticides to control damage caused by insects and diseases with the ultimate goal of increasing crop yields. Oil producing crops such as groundnut, corn, soybeans, mustard and sunflowers demand a wide range of insecticides such as organophosphorus, organochlorine, carbamate etc. and fungicides such as phthalimides, triazines, sulphamide etc. Even with thorough harvesting cleanup, pesticide residues may still occur in the final edible oil products. The growing public concern about the potential health risks posted by the presence of pesticides residues in the human diet has substantially impacted crop protection strategies, which now focus on food quality and safety. Accordingly, pesticides usage is subject to strict regulations, especially with regard to residual levels in commercial goods. The Codex Alimentarius Commission of the Food and Agriculture Organization (FAO), the World Health Organization (WHO) and Food Safety Authority of India have established maximum residue limits (MRLs) in oils for a large number of pesticides. The MRLs in edible oil are usually in the order as 10 µg/kg or higher. In addition, the new emerging field of “organic food” requires the testing methods to analyze pesticides residue at very low levels (i.e. 0.5–2 µg/kg).

2.2 The detection of pesticides in food usually includes the combination of LC–MS/MS and GC–MS/MS, where LC–MS/MS provides sensitive and reliable analysis for more polar to intermediate polar pesticides, and GC–MS/MS provides anlysis on intermediate polar to non-polar pesticides detection where compounds are usually neutral and difficult for ionization by LC–MS/MS. The analysis of lipophilic pesticides is critical in fatty matrices like oil since lipophilic or fat-soluble pesticides tends to accumulate in oil during its production. It was demonstrated that fat soluble pesticides can be concentrated in oils from the oilseeds 1.5–5 times. In addition to the concentrating effect of lipophilic pesticides in oil, the oil matrix also stabilizes and protects these compounds from degradation or oxidation even at low concentrations.

2.3 The development of multiresidue methods for the determination of hydrophobic pesticides in edible oil at low levels is still a challenging issue. Significant effort has been put on the extraction of hydrophobic pesticides from sample matrices and cleanup of lipids prior to analysis. An efficient cleanup of the oil extract is necessary to

improve column lifetime and reduce the instrument downtime due to maintenance. For example, inefficient sample cleanup can allow matrix co-extractives to deposit on the instrument flow path and therefore decrease analyte sensitivity by deteriorating the flow path inertness. It is also challenging to remove interfering lipids without removing the fat-soluble pesticide classes such as organochlorine (OC).

2.4 The sample preparation involves extracting the target analytes from the oil sample and removing co-extracted components from the matrix. To date, liquid-liquid extraction (LLE) is a primary technique used for pesticides extraction from oil matrices. Commonly used solvents include hexane saturated with acetonitrile or petroleum ether. LLE can provide efficient extraction for non-polar pesticides, but it also extracts high levels of co-extractives, which complicates the subsequent cleanup steps. Another common approach is to treat the oil extract with gel permeation chromatography (GPC) or SPE purification using florisil cartridges. These techniques are labour intensive, time consuming and require large amount of hazardous solvents. The Quick Easy Cheap Effective Rugged and Safe (QuEChERS) extraction technique is another popular method, where oil samples are mixed with acetonitrile and water followed with salt induced phase separation. The initial QuEChERS extract is further cleaned by dispersive solid phase extraction (dSPE) where the extract is mixed with different sorbent combinations such as primary-secondary amine + C18 (PSA + C18) and anhydrous $MgSO_4$. Lipid freeze out prior to dSPE can be used for further removal of lipids from oil extracts. The QuEChERS extraction provides cleaner extracts than liquid extraction, but due to the use of the moderately polar solvent acetonitrile and the addition of water during salt partition, the extraction efficiency for non-polar analytes is limited. Traditional dSPE cleanup struggles to effectively and selectively remove lipids while lipid freezing-out requires a significant amount of time and can disrupt analyte solubility. These cleanup techniques are non-selective for lipid removal resulting in the loss of non-polar analytes during samples cleanup. Additionally, the limited oil matrix removal by traditional dSPE cleanup also resulted in higher sample dilution being used, which either required a concentrating step to correct sample dilution, or raised detection limit in oil. Other sample preparation and cleanup techniques used for oil preparation include matrix solid-phase dispersive (MSPD), microwave-assisted extraction (MAE), pressurized liquid extraction (PLE), headspace solid phase micro-extraction (HS-SPME), and supercritical fluid extraction (SFE). These techniques/methods can require large amounts of time, increase complexity, or require specialized equipment.

2.5 Considering the above constraints in the existing multiresidue extraction methods for the non-polar pesticides, the panel decided to validate the recently developed multiresidue methods for edible oils by EU countries using new clean-up technique.

3. Objective of the Project

3.1 The currently used multiresidue QuEChERS method for edible oils in EU countries use EMR-Lipid based cleanup technique. The EMR-Lipid dSPE cleanup tube was introduced in 2015. This method involves mixing sample extract mixture with 1 g of loose EMR-Lipid dSPE sorbent in a 15 mL tube followed with centrifugation. The EMR-Lipid dSPE sorbent selectively interacts with lipids that have long unbranched

hydrocarbon chains, leaving “bulky” target analytes in solution for subsequent analysis making it ideal for multi-class, multi-residue analysis. Previous studies investigated multi-class analysis pesticides and veterinary drugs in fatty matrices and provided high matrix cleanup as well as high analyte recovery and precision in samples including edible oils. The EMR-Lipid dSPE requires mixing ACN sample extract with water at a ratio of 1:1, in order to achieve the desired lipids removal function. Using this mixture is not ideal for hydrophobic pesticides such as organochlorine due to the poor solubility in solvent mixtures containing 50% water. In 2017, EMR-Lipid cartridges and plates were developed, offering a pass-through cleanup. EMR-Lipid cartridges and plates only requires up to 20% water in sample mixture to obtain equivalent lipids removal efficiency as its predecessor. This improvement improves hydrophobic analyte solubility during the sample preparation procedure.

3.2 This study investigates the sample preparation using EMR-Lipid cartridge pass-through cleanup for the analysis of 46 representative and challenging pesticides by GC–MS/MS in edible oils, including groundnut oil, mustard oil, olive oil, corn oil, soybean oil and canola oil. The method can estimate around 125 pesticides from edible oils using this new cleanup technique by LC-MS/MS and GC-MS/MS instruments.

3.3 To validate the newly established multiresidue QuEChERS method using EMR-Lipid sorbent for edible oils.

4. Scope of the Study

4.1 To study the current literature pertaining the QuEChERS cleanup adsorbent for oil extract.

4.2 The method needs to be verified at least for edible palm oil, soyabean oil, rapeseed oil, mustard oil, and groundnut oil.

4.3 At least 150 pesticides comprising moderately polar and non-polar compounds should be tested. In order to select the pesticides, it is advisable to study the monitoring residue data for market survey and registered pesticides for the concern oilseeds.

4.4 To perform the preliminary study for method performance by at least three laboratories having NABL-17025 accreditation with edible oils as a scope of the accreditation.

4.5 After finalisation of the performance of the residue method in different edible oils, method validation as per the SANTE guideline should be performed in at least three NABL-17025 accredited laboratories.

5. Research Methodology

5.1 Single Laboratory Validation and Method Performance

5.1.1 Current literature on the proposed residue method should be reviewed thoroughly. Also, the regulatory method used in different countries should be referred.

5.1.2 Study the label claim of pesticides for different oils seeds in India and the country of import.

5.1.3 Based on the national monitoring data regarding the detection of pesticides in oils and oil seeds, a list of pesticides can be prepared to include in the scope of analysis.

5.1.4 Type of edible oils should be palm, soyabean, rapeseed, mustard and groundnut oils.

5.1.5 The appropriate multiresidue QuEChERS method adopting EMR-Lipid adsorbent needs to be selected for single laboratory validation as per the SANTE guideline. The validation should demonstrate the identity and concentration of the analyte, taking into account for matrix effects, provide a statistical characterization of recovery results, and indicate if the frequency of false positives and negatives are acceptable. When the method is followed using suitable analytical standards, results within the established performance criteria should be obtained on the same or equivalent sample material by a trained analyst in any experienced residue testing laboratory. To ensure method performance remains appropriate over time, method validation should be continuously assessed (e.g. recovery spikes).

5.1.6 After validation, the method documentation should provide, in addition to performance criteria (data quality objectives), the following information:

(a) Identity of the analytes included in the residue definition.

(b) Concentration range covered by the validation;

(c) Matrices used in the validation;

(d) Protocol describing the equipment, reagents, detailed step-by-step procedure including permissible variations (e.g. “heat at 100 ± 5 °C for 30 ± 5 min”), calibration and quality procedures, special safety precautions required, and intended application and critical uncertainty requirements;

(e) quantitative result of the expanded measurement uncertainty (MU) for the method should be calculated in the validation procedure and reported, if required.

5.1.7 Selectivity: As a general principle, selectivity should be such that interferences have no impact on method performance. The ultimate test of selectivity involves the rates of false positives and negatives in the analyses. To estimate rates of false positives and negatives during method validation, an adequate number of blanks per matrix [not from the same source] should be analysed along with spiked matrices at the analyte reporting level.

5.1.8 Calibration: There are some characteristics of calibration that are useful to know at the outset of method validation because they affect optimization of the final protocol. For example, it must be known in advance whether the calibration curve is linear or quadratic, passes through the origin, and is affected by the sample matrix or not. The following calibration procedures are recommended for the initial method validation:

(a) determinations at five or more concentrations should be performed (consider multiple injections per concentration);

(b) the reference standards should be evenly spaced over the concentration range of interest and the calibration range should encompass the entire concentration range likely to be encountered;

(c) the reference standards should be dispersed over the whole sequence, or encompass the beginning and end of the run to demonstrate that calibration integrity is maintained over the entire sequence; and the fit of the calibration function must be plotted and inspected visually and/or by calculation of the residuals (differences between the actual and calculated concentrations of the standards), avoiding over-reliance on correlation coefficients. If residuals of the calibration curve deviate by more than ±20 – 30 % (30% for calibration concentrations near the instrument LOQ), statistical consideration of outliers should be made, possibly leading to re-analysis of the sequence if quality control criteria are not met.

5.1.9 Linearity: Linearity can be tested by examination of a plot of residuals produced by linear regression of the responses on the concentrations in an appropriate calibration set. Any curved pattern suggests a lack of fit due to a nonlinear calibration function. If this is the case, another function such as quadratic should be tested and applied, using at least five concentration levels. Despite its current widespread use as an indication of quality of fit, the coefficient of determination (R^2) may be misleading because it places greater significance on standards with higher concentrations. In this case, an appropriate weighting factor such as 1/x or 1/x2 should be considered to minimize the potential impact of the relative concentration range.

5.1.10 Matrix Effects: Matrix-matched calibration is commonly used to compensate for matrix effects. Extracts of blank matrix, preferably of the same or similar type as the sample, should be used for calibration. If solvent-only calibration is used, a measurement of matrix effects must be made to demonstrate equivalence of results by comparing responses of matrix-matched with solvent-only standards.

5.1.11 Trueness and Recovery: Trueness is the closeness of agreement between a test result and the accepted reference value of the property being measured. Trueness is stated quantitatively in terms of “bias,” with smaller bias indicating greater trueness. Recovery refers to the proportion of analyte determined in the final result compared with the amount added (usually to a blank) sample prior to extraction, generally expressed as a percentage. Errors in measurement will lead to biased recovery figures that will deviate from the actual recovery in the final extract. Routine recovery refers to the determination(s) performed in quality control spikes in the analysis of each batch of samples.

5.1.12 Precision: Precision is the closeness of agreement between independent (replicate) test results obtained under stipulated conditions. It is usually specified in terms of standard deviation (SD) or relative standard deviation (RSD), also known as coefficient of variation (CV). For single-laboratory validation, two types of precision sets of conditions are relevant: (a) repeatability, the variability of measurements within the same analytical sequence, and (b) within-laboratory reproducibility, the variability

of results among multiple sets of the same sample. The initial validation should be conducted at the targeted limit of quantification (LOQ) or reporting limit of the method, and at least one other higher level, for example, 2-10x the targeted LOQ or the MRL.

5.1.13 Limit of Quantification (LOQ): By long-standing definition among analytical chemists, the LOQ is the concentration at which the average signal/noise ratio (S/N) equals 10 in the analysis. The LOQ in practice can only be estimated because precise determination of the actual LOQ requires many analyses of spiked samples and matrix blanks but the LOQ can change day-to-day due to the performance state of the instrument, among many other factors. Some validation guidelines require that the LOQ be verified to meet method performance criteria via spiking experiments at the LOQ, however day-to-day variations in LOQ tend to force the analyst to greatly overestimate the actual method LOQ, which can be difficult to implement the strict definition of the LOQ (S/N = 10). Thus, spiking at the Lowest Validated Level (LVL) is the more descriptive and proper approach. Furthermore, quantification of analytes should not be made below the lowest validated level (LVL) in the same analytical sequence. The S/N at the lowest calibrated level (LCL) must be ≥10 (conc. ≥ LOQ), which can be set as a system suitability check required for each analytical sequence. A quality control matrix spike can also be included in each sequence to verify that the reporting limit is achieved in the analysis (an action level that is typically >the LCL). In essence, the point of the validation is not to determine the LOQ, but to demonstrate that the lowest reported concentration is meeting the need for the analysis. While not useful for quantification, some analysts may wish to calculate the limit of detection (LOD) (S/N = 3) to infer the presence of the analyte at concentrations too low to permit an estimate of analyte concentration.

5.1.14 Analytical Range: The validated range is the interval of analyte concentration within which the method can be regarded as validated. The LVL is the lowest concentration assessed during validation that meets method performance criteria. It is important to realize that the validated range is not necessarily identical to the useful range of the instrumental calibration. While the calibration may cover a wide concentration range, the validated range (which is usually more important in terms of uncertainty) will typically cover a more restricted range. In practice, most methods will be validated for at least two levels of concentration.

5.1.15: Ruggedness: The ruggedness (often synonymous with robustness) of an analytical method is the resistance to change in the results produced by the analytical method when deviations are made from the experimental conditions described in the procedure. Examples of the factors that a ruggedness test could address are: small changes in the instrument, brand/lot of reagents or changes in operator; concentration of a reagent; pH of a solution; temperature of a reaction; time allowed for completion of a process, and/or other pertinent factors.

5.1.16 Measurement Uncertainty (MU): The formal approach to measurement uncertainty estimation is a calculated estimate from an equation or mathematical model, around which the true value can be expected to lie within a defined level of probability. It is preferable to express the uncertainty of measurement as a function of

concentration and compare that function with a criterion of fitness for purpose agreed between the laboratory and the client or end-user of the data.

5.2 Collaboration:

5.2.1 Number of Participating Laboratories: Minimum three laboratories with NABL-17025 accreditation. After method validation, a robust protocol should be adopted by even more laboratories to generate the results with minimum variation.

6. Deliverables

Detailed project report of the work done, in hard copy and digital formats, as per the scope specified under 4, with the following as appendices:

a) Research findings and data collected regarding the development of QuEChERS based multiresidue method and its validation.

b) Test results generated during the development and validation of the test method

7. Timeline and Method of Progress Review

7.1 Timeline for the project is 6 months from the date of award of the project

7.2 Stages of review:

Stage	Timeline
Stage I: Primary Report covering the review of the literatures and complete plan regarding the development of the test method as per the scope.	15 days
Stage II: Purchase of the chemicals, reagents, and other required consumables for the validation of the method	One month
Stage III: Validation of QuEChERS based multiresidue method for different oils.	Two months
Stage IV: Performance of the method by different laboratories.	Two months
Stage V: Preparation of final protocol with all the validation and method performance data.	15 days

At the end of 6th month, project allottee to submit final project report incorporating recommendations/feedback of Committee

Note: *The timelines given above are indicative and calculation of time will start from the date of award of sanction letter for the project to the Project leader.*

8 Support from BIS

8.1 Access to Indian and International Standards

8.2 Letters from BIS to concerned stakeholders for support in research project.

9. Nodal Officer