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A Modified and Validated QuEChERS Method for Agrochemical Residue Analysis in *Nicotiana tabacum* Leaf Using Green Reagents (Ionic Liquids and Nanocarbons)

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A modified QuEChERS multi-residue method using green reagents (*Nicotiana tabacum* waste stems-generated nanocarbons and ionic liquids) was optimized and validated for *N. tabacum* samples. Instrumental analysis was carried out using liquid chromatography tandem mass spectrometer. 1-Benzyl-3-methylimidazolium chloride was established to be the ideal ionic liquid to replace the traditional organic solvent and 10 ml of this was the optimum volume. The ideal nanocarbon weight was found to be 20 mg. The limit of detection ranged from 0.006 $\mu g g^{-1}$ to 0.025 $\mu g g^{-1}$ and limit of quantification ranged from 0.01 $\mu g g^{-1}$ to 0.05 $\mu g g^{-1}$ with relative standard deviation ranging from 12.55% to 19.98% for the seven analytes studied. The recoveries for the analytes ranged from 69.9% - 120.1% with RSD of less than 18%. The expanded uncertainty at 95% confidence using a coverage factor (k = 2) was found to be 5.10%. The validated method was successfully applied for the determination of methamidophos and monocrotophos in real *N. tabacum* samples from selected tobacco-growing regions in Zimbabwe.

Key words: *N. tabacum*; nanocarbons; ionic liquids; green reagents; method validation; liquid chromatography tandem mass spectrometer; 1-Benzyl-3-methylimidazolium chloride; methamidophos; monocrotophos

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Nicotiana tabacum plant is sensitive to various diseases and pests [1]. Its production in Zimbabwe heavily relies on the use of agrochemicals which must be registered for use [2]. To use environmentally friendly agrochemicals, certain agrochemicals such as 'purple and red' labelled agrochemicals have been discontinued for *N. tabacum* production. These agrochemicals however, need to be monitored for abuse since they have been known to leave unwanted residues in the crop. The discontinued agrochemicals include acephate, aldicarb, aldicarb sulphoxide, aldicarb sulphone and methamidophos. The discontinued agrochemicals have adverse effects on human health as well as on the environment, a phenomenon which have been explicitly noted in the second-half of the twentieth century [3].

N. tabacum contains complex plant components such as fatty acids, lipids, sugars, pigments, alkaloids and phenols [4]. Analysis of multi-agrochemical residues in *N. tabacum* is difficult due to matrix interferences. Generally, analysis of pesticide residues entails extraction of analytes from a sample matrix, clean-up and pre-concentration and finally instrument separation and determination. Various techniques for clean-up, and extraction processes have been reported and these include gel permeation chromatography (GPC), solid-phase extraction (SPE), solid-phase microextraction (SPME), stir-bar sorption extraction (SBSE), microwave- assisted extraction (MAE), dispersive solid-phase extraction (dSPE), matrix-assisted solid-phase dispersion (MASPD), liquid-liquid extraction (LLE), and dispersive liquid-liquid extraction (dLLE) [4, 5]. Solid phase extraction using different types of sorbents such as graphitised carbon black (GCB), primary secondary amine (PSA) and florisil or a mixture of these, using organic solvents have been reported [6]. The complex *N. tabacum* matrix presents high co-extractive content making it difficult to remove these pigments and other non-polar interfering substances when using small organic solvent quantities used in SPE. Green chemistry approach has resulted in the shift from traditional methods which are labour intensive and solvent consuming to safer, fast and efficient methods. QuEChERS original method was developed by Anastassiades and modified versions of it were developed [7, 8, 9] to alleviate the above issues. This method makes use of rigorous extraction process using small quantities of extraction reagents.

Nanocarbons have found use in biotechnology, pharmaceutical and analytical chemistry fields among others [3, 10, 11, 12, 13]. Nanocarbon materials' high surface area has made them useful specifically in

analysis of agrochemicals, a distinct advantage to remove the complex *N. tabacum* matrix applying them to QuEChERS extraction process [12]. This advantage stems from the fact that in agrochemical analysis, the sorbent binds sample matrix compounds rather than analytes of interest and it is based on dSPE.

The introduction of ionic liquids has sparked a growing interest in their application in analytical chemistry. They are unique solvents which are less environmentally damaging than organic solvents and are useful in reducing levels of environmental pollution [5]. They exhibit good solubility towards organic and inorganic compounds and are not volatile [14]. They are not made of molecules but are made up of ions present in liquid as positive and negative ions with exactly equal amounts of each making the whole liquid electrically neutral [15].

In this study, a novel modified QuEChERS method was developed and validated using green reagents (nanocarbons and ionic liquids) in the extraction and analysis of several agrochemicals from complex *N. tobacco* matrix samples. Parameters that impact extraction efficiency of this method was optimised, including the type and volume of ionic liquid [IL], and nanocarbons weights.

METHODS

Chemicals and Reagents

Analytical standards (acephate, aldicarb, aldicarb sulphoxide, aldicarb sulphone, methamidophos, metolachlor, and monocrotophos) of >99% purity, Ionic liquids [1-Benzyl-3-methylimidazolium hexafluorophosphate (ILA), 1-Benzyl-3-methylimidazolium tetrafluoroborate (ILB) and 1-Benzyl-3-methylimidazolium chloride (ILC)], methanol and acetonitrile reagents were purchased from Sigma Aldrich, Germany. Nanocarbons were generated in the Tobacco Research Board laboratory. QuEChERS kits were obtained from Agilent Technologies in South Africa. Deionized water was obtained from the Tobacco Research Board.

Instrument and Apparatus

An AB Sciex 5500 model tandem mass spectrometer (MS/MS) equipped with 1.6.2 Analyst software version was coupled to an Agilent liquid chromatography (LC) 1260 series system and used for instrument analysis. The Agilent LC system was made up of the **v**acuum degasser GG1379 B, binary pump G1312B, auto-sampler G1367C, column oven G1316B, analytical column: phenomenex synergi 2.5 µ Fusion-RP 100 Å, 50×200 mm and guard column (phenomenex security guard cartridge with Fusion-RP 4×2.00 mm cartridge). Mass spectrometry was performed using electron spray ionization. A vortex mixer 560500 model manufactured by Pro Scientific Inc. and centrifuge INF1200R model manufactured by Nuve were used for dSPE sample preparation.

Nicotiana tabacum Sample Preparation

Optimized parameters (10 ml of 1- Benzyl-3-methylimidazolium chloride (ILC) and 20 mg nanocarbons) were then used in QuEChERS dSPE process. Preparation of the nanocarbons was reported in our previous work [16], and these were characterized using FTIR, XRD, SEM, TEM and BET before use. The dSPE was then carried out by first weighing 2 g of blank ground (to mesh size ~ 2 mm) cured leaf *N. tabacum* samples into plastic centrifuge tubes, spiked with the spiking standard of 1×10^{-6} g/ml before adding 10 ml of ILC reagent. The mixture was vortexed for 1 min and then centrifuged at 4200 rpm for 5 minutes. 1.5 ml aliquot sample was taken for dSPE analysis. The aliquot was placed in a 2 ml plastic tube containing 20 mg of nanocarbons prepared in the laboratory, 40 mg of C_{18} sorbents, 50 mg of $MgSO_4$ and 25 mg of PSA, vortexed for 1 min and centrifuged for 5 min at 4200 rpm. 0.8 ml of the vortexed mixture was then filtered through a 0.45 µm filter into an instrument vial and 0.8 ml of mobile phase A (5 ml of 1 M ammonium formate solution was added to 895 ml of deionised water and 100 ml methanol) added to the sample. This was agitated and analysed by LC-MS/MS. Samples were spiked at different levels for recoveries before processing.

The method was validated and uncertainty of measurement established. The technique was then used on real cured *N. tabacum* leaf ground samples taken from routine samples brought into the laboratory by clients to demonstrate the applicability of the nanocarbons as dSPE sorbent for sample clean-up and ionic liquid reagent on these classes of pesticides.

RESULTS AND DISCUSSION

During the optimization of dSPE several factors affecting the extraction efficiency of the dSPE were examined including the effect of sorbents, type of ionic liquid, the volume of ionic liquid and amount of nanocarbons used.

Performance of C_{18}, PSA and Nanocarbons Sorbents

Figures 1, 2 and 3 show the retention times in minutes of the analytes versus their intensity in counts per second of the analytes. Figures 1 and 2 show that the PSA and C_{18} sorbents managed to remove co-extractives which although do not affect analyte

retention times and recoveries, may affect the lifetime of instrument columns and the instrument. PSA removes sugars and fatty acids while C_{18} removes non-polar interferences such as lipids. The introduction of nanocarbons resulted in cleaner chromatograms as shown in Figure 3 with no effect on recoveries as shown in Figure 10.

Evaluation for Extraction Solvent Compatibility

The selection of extraction solvent plays a critical role in directly determining the extraction efficiency of a method. The solvent characteristics entail good extraction capability for analytes of interest, low volatility, good analyte solubility, good chromatographic behaviour with no interference with analyte peaks of interest. In this experiment, an organic solvent (acetonitrile) and the three ionic liquids were compared for analyte solubility and instrument compatibility. All the analytes had good solubility for the selected agrochemicals and were compatible with the LC-MS/MS instrument as exhibited by detection of all the analytes and stable instrument responses for analytes in blank solutions. The instrument was able to detect the analytes and no interferences with the analytes of interest were observed.

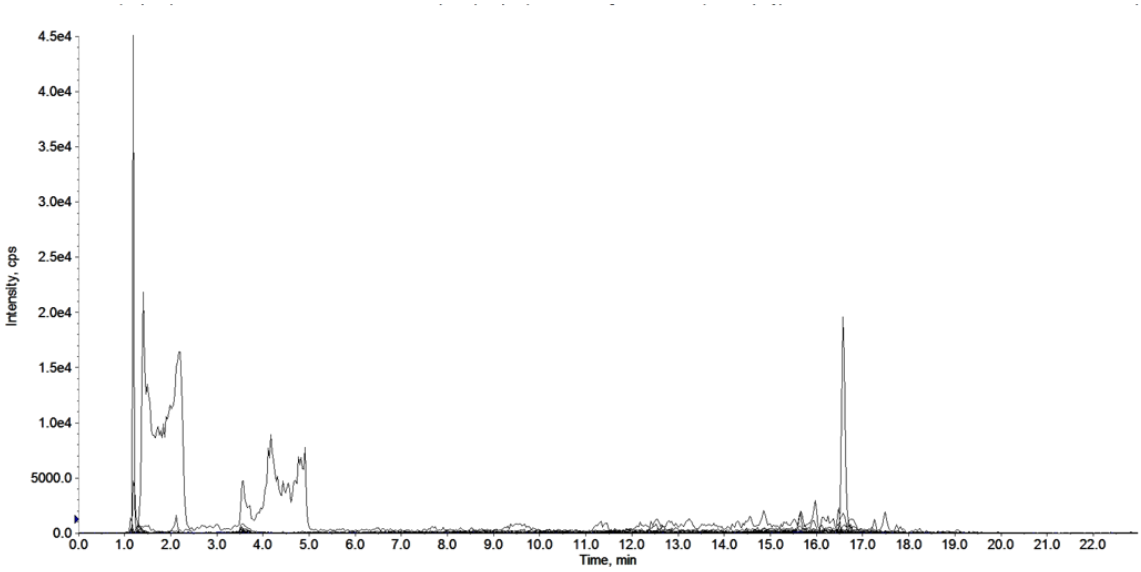

Figure 1. *N. tabacum* sample dSPE with C_{18} sorbent.

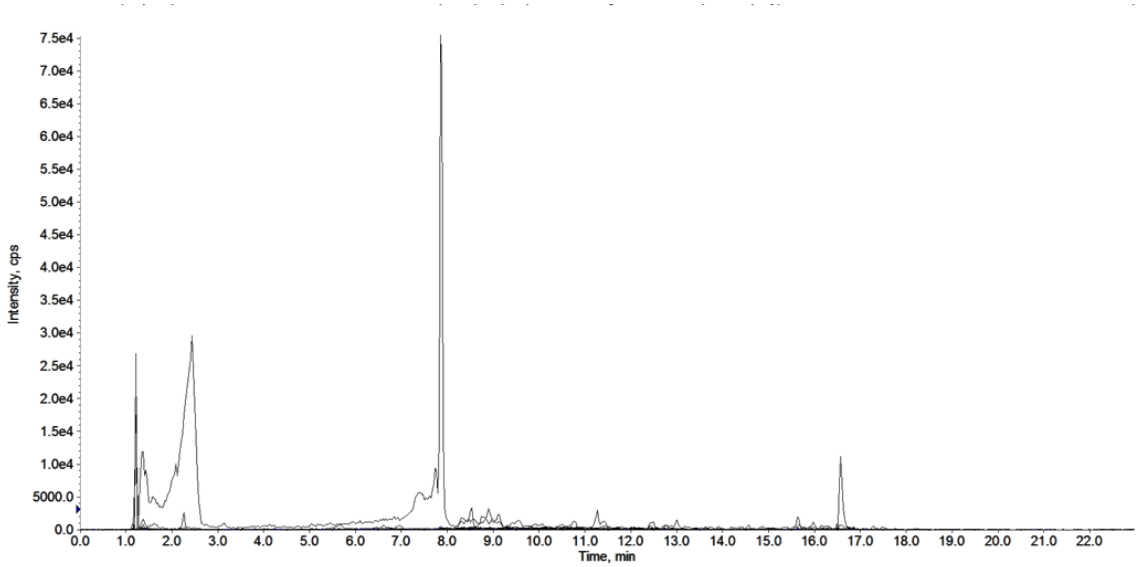

Figure 2. *N. tabacum* sample dSPE with C_{18} and PSA sorbents.

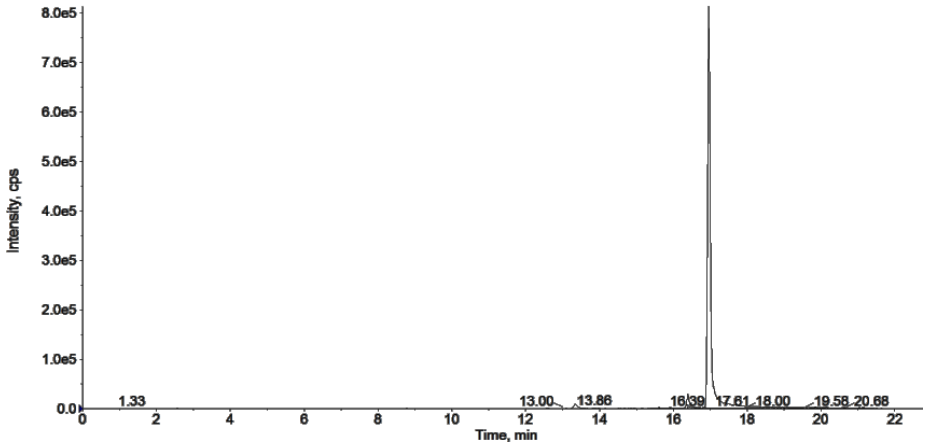

Figure 3. *N. tabacum* sample dSPE with $C_{18,}$ PSA and nanocrbons sorbents.

Solvent Selection Optimisation

Figure 4 shows that ionic liquid C have a comparable performance to the traditional acetonitrile organic solvent normally used in the QuEChERS extraction process but which is not environmentally friendly. ILC was selected for further trials. Low recoveries by ILA and ILB may be due to stronger hydrophobic interactions with the nanocarbons compared to acetonitrile and ILC. ILC gave acceptable results [15] ranging between 70% and 120% for five analytes (Figure 4). Although methamidophos and mono-crotophos each gave 60% recoveries for organic solvent results, overall results were accepted because of good precision for both analytes.

Evaluating the Effect of Volume of Ionic Liquid (ILC)

Comparison of recoveries was also carried out to establish optimum extraction solvent volume. The volume of solvents used ranged between 10 ml and 40 ml of ILC. A volume of 10 ml was then chosen since it gave comparable recoveries for all analytes with consistent results and was economical in terms of quantities required (Figure 5).

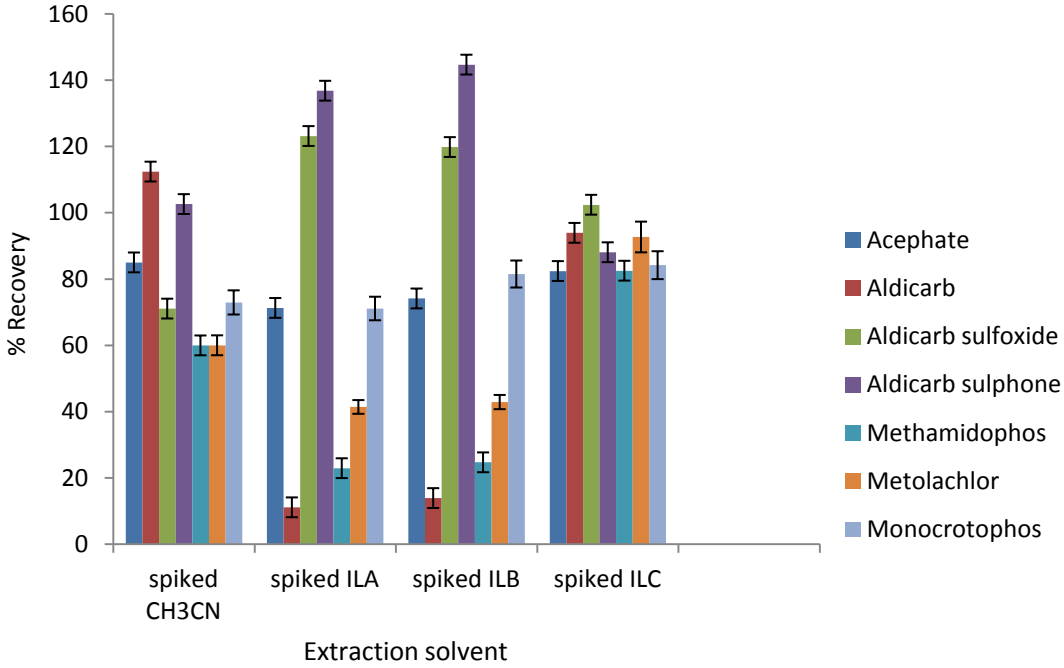

Figure 4. Comparison of spiked acetonitrile and the three ionic liquids neat reagents (n = 3). Error bars represent the standard deviation.

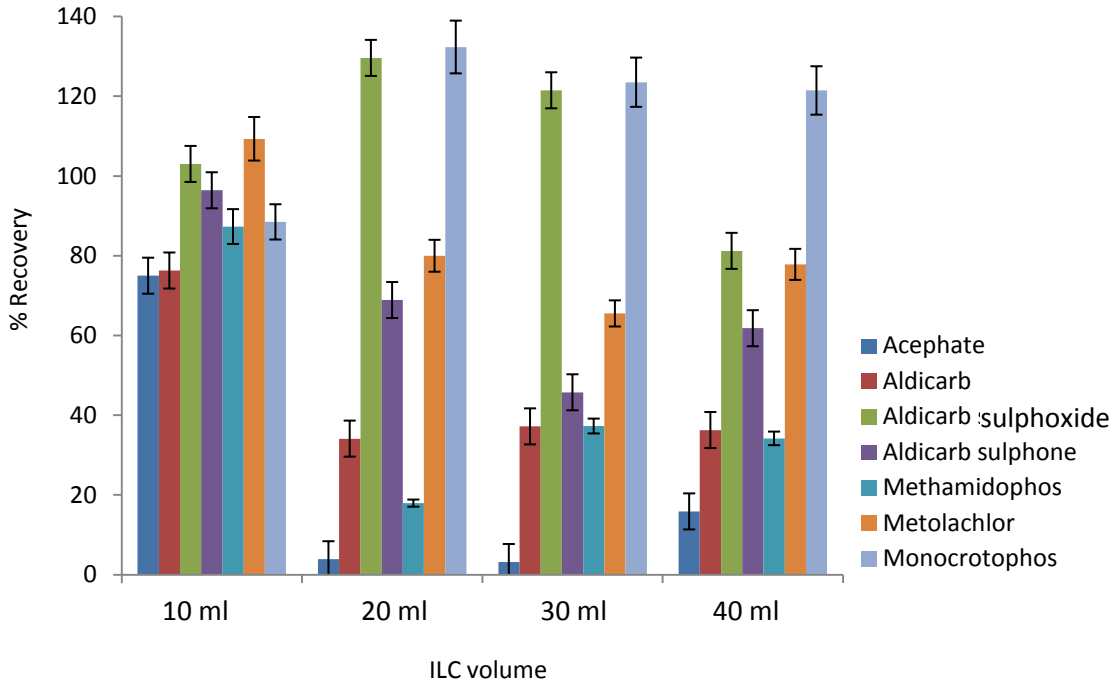

Figure 5. Evaluating effect of ILC volume on sample extraction (n = 3). Error bars represent the standard deviation.

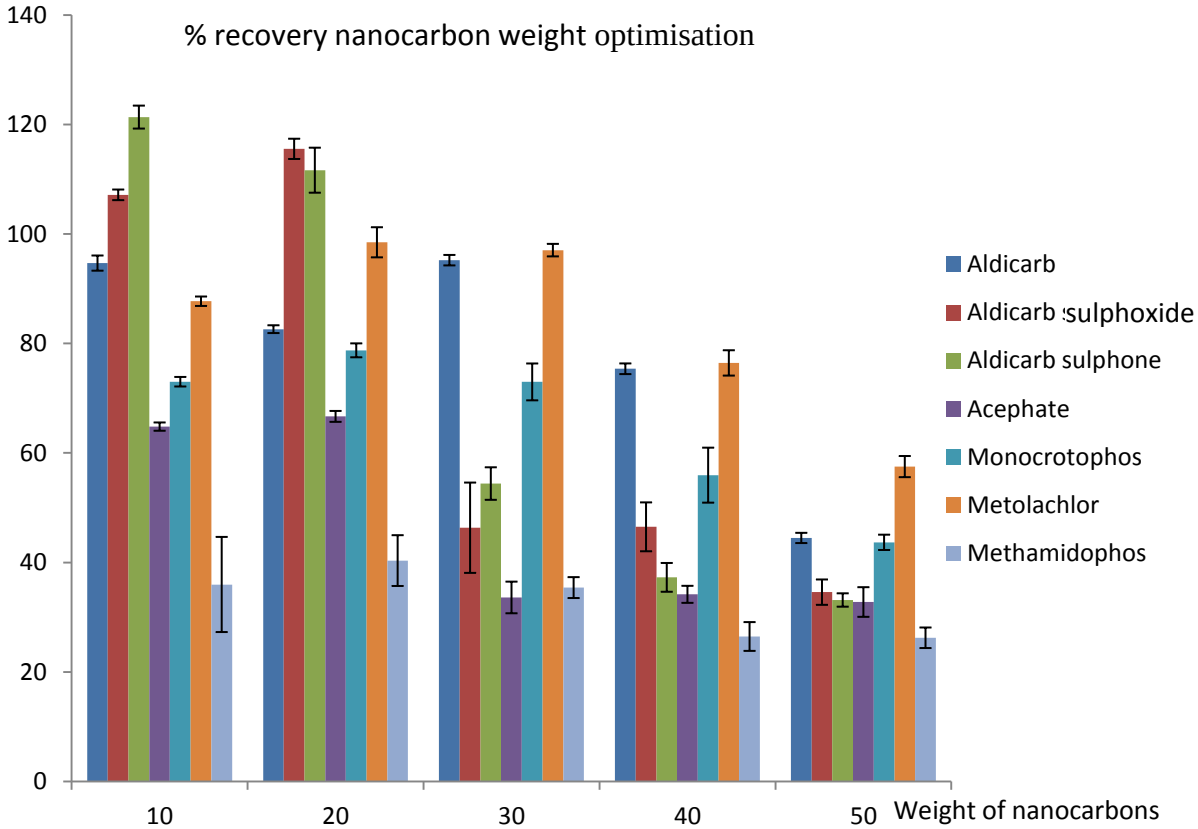

Figure 6. Evaluating effect of varying the weights of nanocarbons on spiked samples on sample clean-up (n = 3). Error bars represent the standard deviation.

This volume is similar to the optimum volume used in the conventional QuEChERS method which uses organic solvents [8]. From this trend, dilution seems to make the analytes bind more to the sorbent thereby being unavailable for determination. However, for highly polar acephate and methamidophos, recoveries of these analytes are reduced by dilution. This is contrary to expectation, possibly because the ionic solvent is being used in place of the traditional organic solvent. Aldicarb converts to aldicarb sulphoxide and aldicarb sulphone metabolites. This explains the observed increased trends in recoveries for these metabolites with an increase in volume.

Nanocarbon Weight dSPE Optimization Process Using Ionic Liquid C

Weights of the sorbent can have an impact on the clean-up ability of the sorbent. One distinct characteristic for using nanocarbons in this work is their large surface area which is ideal for cleaning the *N. tabacum* matrix. Different masses of nanocarbons were evaluated ranging from 10 mg to 50 mg. Recoveries were noted to generally increase from 10 mg to 20 mg (Figure. 6). Recoveries started to decrease, as nanocarbon content increased from 30 mg to 50 mg. Increasing the amount of adsorbent enhanced the amount of analyte retained on the sorbent leaving fewer analytes in solution. The agrochemical group under study formed some π- π interactions with the nanocarbons. Increase in nanocarbon content resulted in the observed trend. Therefore, 20 mg was then finally chosen as the optimum weight of adsorbent for use.

Method Validation and Uncertainty of Measurements

The optimum dSPE method entails extraction of 2 g ground *N. tabacum* dried leaf sample and extracting with 10 ml of ILC and clean up with PSA combined with 20 mg of the prepared nanocarbons followed by LC-MS/MS instrument analysis of the selected seven analytes.

METHOD VALIDATION

Linearity Range, Correlation Coefficients (R^2), LOD and LOQ (n = 10) of the Analytes

Satisfactory correlation coefficients (R^2) for the seven analytes were observed ranging from 0.992-0.9973 for linearity ranges as shown in Table 1. Sensitivity was established using limit of detection (LOD), limit of quantification (LOQ) and standard deviation from the same table. The limit of detection is the lowest detectable amount of analyte in a sample which can be detected but not necessarily quantified as an exact value. It is obtained as a signal of the blank plus three times the standard deviation of the blank while the LOQ is the lowest quantifiable amount of analyte and it is the signal of the blank plus ten times the standard deviation of the blank (all for ten replicates).

Accuracy

The accuracy of a method is the closeness of the mean results obtained to the actual value of the analyte.

Table 1. Linearity range, correlation coefficients (R^2), LOD, LOQ (n = 10) of the analytes.

Peak	Analyte	Linearity range (μgg^{-1})	R^2	LOD (μgg^{-1})	LOQ (μgg^{-1})	δ
1	Acephate	0.3 - 2.5	0.9941	0.025	0.05	0.003144
2	Aldicarb	0.3 - 2.5	0.9922	0.009	0.02	0.001020
3	Aldicarb sulphoxide	0.3 - 2.5	0.9973	0.006	0.01	0.0005100
4	Aldicarb sulphone	0.3 - 2.5	0.9927	0.011	0.02	0.001128
5	Methamidophos	0.05 - 1.00	0.9936	0.014	0.03	0.001668
6	Metolachlor	0.3 - 2.5	0.9923	0.016	0.03	0.001984
7	Monocrotophos	0.05 - 1.00	0.9940	0.009	0.02	0.001102

It can be expressed as an absolute error, relative error or as percentage of trueness. To test for accuracy, replicate blank *N. tabacum* samples were spiked at different spiking levels (1 μgg^{-1} and 0.5 μgg^{-1} ppm) of a mixed standard containing the seven analytes of interest and recoveries calculated. This recovery ranged from 69.9% to 120.1% for the seven analytes with RSD ranging between 1.46% and 18.06%. A *t*-test for bias was also carried out on the results to establish the differences between the two means. $T_{cal} < T_{tab}$, for acephate, aldicarb, aldicarb sulphoxide , aldicarb sulphone and methamidophos suggesting that at 95% confidence level, there are no significant differences between the results mean for 0.5 μgg^{-1} and results mean for 1.00 μgg^{-1} for acephate, aldicarb, aldicarb sulphoxide , aldicarb sulphone, and methamidophos. However, $T_{cal} > T_{tab}$ for metolachlor and mono-crotophos, implying there were significant differences between the results mean for metolachlor and monocrotophos for 0.5 μgg^{-1} and 1.00 μgg^{-1} spiking levels. The calculated means for metolachlor and monocrotophos suggest that the results were accurate as they fell between the 70% and 120% recoveries which are acceptable [17].

Precision

The precision of a method expresses the closeness in agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions. It can be expressed as repeatability, intermediate precision or reproducibility. In this research repeatability and intermediate precision were evaluated.

Repeatability

Repeatability expresses the precision under the same operating conditions over a short period. It is also known as intra-assay precision. Ten analysis of the same homogenous sample (spiked at 1.00 μgg^{-1}) were analysed to test for repeatability. The results showed that RSD obtained is less than 20% for the seven analytes implying the results are repeatable.

Intermediate Precision

This expresses within laboratory variations at different days of analysis. To test for the intermediate precision the variances for two days where determined to see if they were significantly different using the F-test. There was no evidence at 95% confidence level to suggest that there is a difference in precision. Since no significant difference was found, a pooled standard deviation with 18 degrees of freedom was calculated and comparison of the two means for the two days was based on the null hypothesis. The null hypothesis was therefore retained and conclusion made that at 95% significance level, there is no significant difference in the means for the two days.

Measurement Uncertainty

Measurement uncertainty was done using the GUM modeling approach [17]. Data from the in-house validation studies was used in the quantification of the different uncertainty components and the results are tabulated in Table 2. The three significant contributions were:

1. The best available estimate of the overall run-to-run variation
2. The best possible estimate of the overall bias and its uncertainty; and
3. Quantification of any uncertainties associated with effects incompletely accounted for by the overall performance studies.

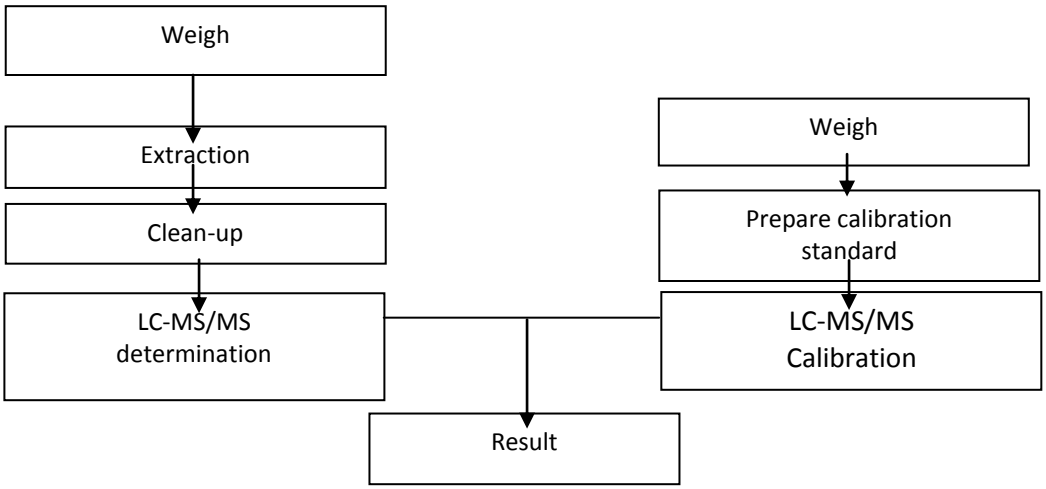

Figure 7. Pesticides analysis in *N. tabacum* process.

Other Sources of Uncertainty

Figure 7 shows the pesticides analysis procedure in *N. tabacum.* All balances and volumetric measuring devices are under regular control and both bias and precision studies incorporate the influence of the uncertainty from these sources. The extent of the variability study took into account environmental factors such as temperature. The purity of the reference standard given by the manufacturer is on average 99.7% ± 0.3%. Therefore purity is an additional uncertainty source with a standard uncertainty of $0.003/\sqrt{3} = 0.00173$ (rectangular distribution). The uncertainty due to reference standards is significant.

Calculation of Combined Standard Uncertainty

The in-house validations of the analytical procedure, the repeatability, the biasness and all other feasible uncertainty sources have been thoroughly investigated. Their values and uncertainties are shown in Table 2. The relative values are combined because the model is entirely multiplicative [18].

Table 2. Uncertainties in agrochemical residue analysis.

Description	Value (x)	Standard uncertainty $u(x)$	Relative standard uncertainty $\frac{u(x)}{x}$	Remark
Repeatability (1)	1	0.0254	0.0254	Duplicate tests of different samples
Bias (REC) (2)	0.996	0.001	0.001	Spiked samples
Standards	1	0.00173	0.00173	Reference standards
Uc			0.0255	Relative standard Uncertainty

$$\text{Relative uncertainty} = Uc = \sqrt{\text{repeatability}^2 + \text{bias}^2 + \text{standards}^2} \quad (1)$$

$$Uc = \sqrt{(0.0254^2 + 0.001^2 + 0.0173^2)} = 0.0255$$

$$Uc = 0.0255 \times 100\% = 2.55\%$$

The expanded uncertainty U is calculated by multiplying the combined standard uncertainty with a coverage factor of 2.

$$U = 0.0255 \times 2 = 0.0510 = 5.10\%$$

The expanded uncertainty at 95% confidence using a coverage factor (k = 2) is 5.10%.

Table 3. Results of real tobacco samples with agrochemical residues.

Sample	Methamidophos (µgg-1)	Monocrotophos (µgg-1)
Sample C1	0.434	<LOQ
Sample C2	0.280	0.370
Sample C3	<LOQ	0.341
Sample E1	<LOQ	0.313
Sample E3	<LOQ	<LOQ

Real *N. tabacum* Leaf Samples Analysed Using the Validated Method

Fifteen *N. tabacum* leaf samples sampled from selected tobacco growing regions in Zimbabwe were analysed for the selected banned agrochemicals. Five of the analysed samples contained a maximum of two analytes residue as indicated in Table 3. This indicates that some of the farmers are still using the banned pesticides in those areas. Results of the other ten samples were below LOQ for all the seven analytes and are not reported in Table 3.

Methamidophos was detected in five samples ranging between 0.280 μgg^{-1} and 0.434 μgg^{-1} while monocrotophos residues were detected in three samples, the concentration ranged between 0.313 μgg^{-1} and 0.370 μgg^{-1}. A 10^{-7} mixed analytes standard was used for quantification. Sample chromatograms are shown in Figures 8-10.

CONCLUSION

In this study, a modified QuEChERS method which uses ionic liquid and *N. tabacum* generated nanocarbons of the nanotubules type characterized using TEM and BET techniques was developed and validated. The method was validated for analysis of seven analytes, acephate, aldicarb, aldicarb sulphoxide, aldicarb sulphone, metolachlor, and methamidophos. The LOD ranged from 0.006 ppm to 0.025 ppm and LOQ ranged from 0.01 ppm to 0.05 ppm with RSD ranging from 12.55% to 19.98% for the seven analytes. The recoveries for the analytes ranged between 69.9% - 120.1% with RSD less than 18.06%. The expanded uncertainty at 95% confidence using a coverage factor (k = 2) is 5.10%. The validated parameters suggested that the method was fit for its intended use and could be successfully utilized to analyze *N. tabacum* leaf samples. The precision, accuracy and optimization results supported by expanded uncertainty indicated

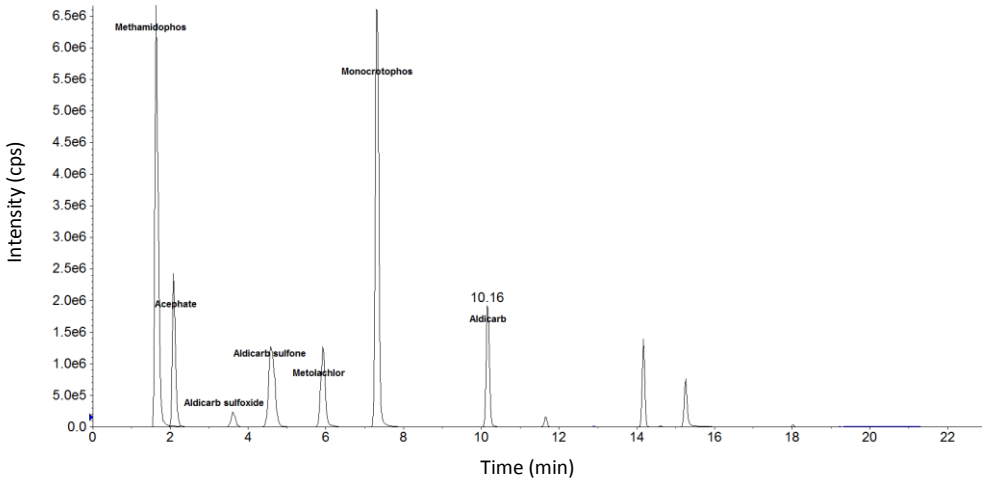

Figure 8. Sample chromatogram of selected banned standards.

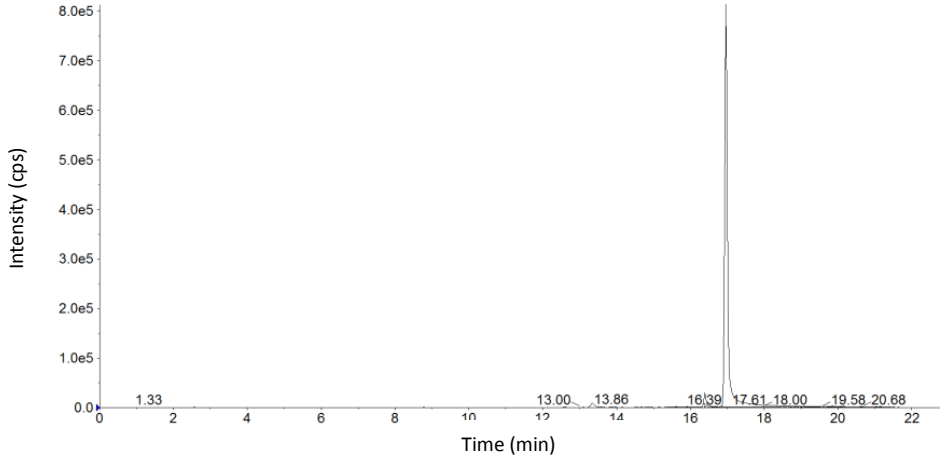

Figure 9. Chromatogram of blank *Nicotiana tabacum* QC sample.

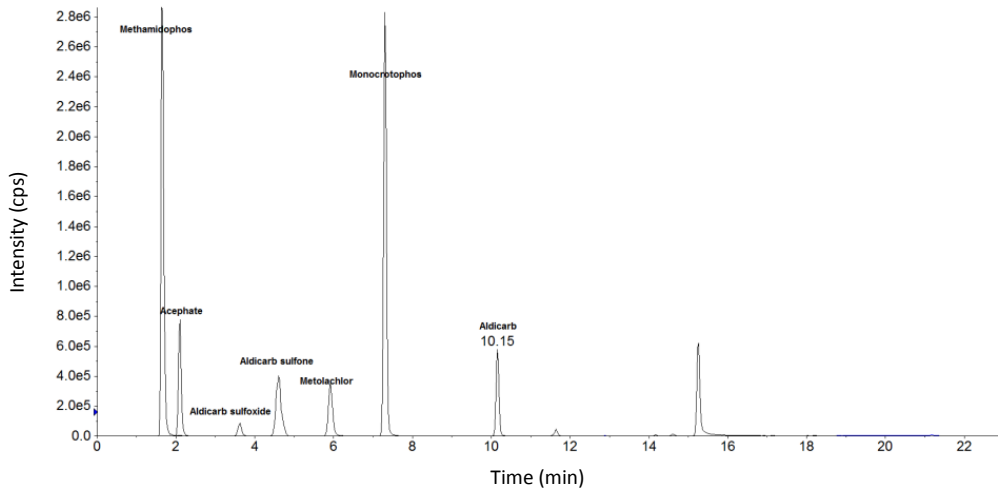

Figure 10. Sample chromatogram of *Nıcotıana tabacum* sample QC spiked at 1 ppm.

that *N. tabacum* generated nanocarbons and ionic liquids green reagents could be used to modify the QuEChERS method for effective analysis.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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