

Application of matrix compatible solid phase microextraction (SPME) coatings for the analysis
in complex food matrices

by

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Author's Declaration

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Abstract

In recent years, studies regarding food analysis have increased due to the globalization of food trade as well as concerns about the safety and traceability of food commodities. In this regard, the field of food analysis has seen a substantial increase in the development of analytical methods used to determine the quality and safety of foodstuffs. The most critical step, regarding food analysis, is the sample preparation step, which is considered the most time-consuming step within the complete analysis and one of the most difficult to automate.

Solid phase microextraction (SPME) is a well-known sample preparation technique that can be easily automated to overcome the tedious and time-consuming sampling-sample preparation step in the field of food analysis. In SPME, one of the most crucial steps in the SPME protocol is the correct choice of a SPME coating for a given application, as its principle of extraction is based on the degree of distribution between the analytes and the sample matrix. Despite its great potential, the applications of SPME in the analysis of complex matrices, such as food, has been on hiatus due to the lack of suitable SPME coatings that possess compatibility with complex matrices while maintaining sufficient sensitivity for the required applications.

Latterly, efforts have been made to the development of coatings that can overcome the issues related to the fouling phenomena, a process that is induced by the matrix components, and that reduces the lifespan of the coatings and affects the extracting selectivity of the analytes directly. One of the recent steps in the development of "matrix compatible coatings" uses one of the most compatible material, the polymer polydimethylsiloxane (PDMS), that used as a coating can present limited extraction efficiency towards less hydrophobic analytes. As an extra layer in the already combined coating that exhibits best extraction efficiency towards contaminants, the polydimethylsiloxane/divinylbenzene (PDMS/DVB) coating, to avoid the attachment of matrix components onto the coating surface, that is a limiting issue for the uses of this solid coating.

Therefore, the present thesis established the application and evaluation of a fully automated solid phase microextraction protocol with a complex food matrix using a PDMS/DVB overcoated PDMS fiber. Furthermore, the evaluation of a new automated station was investigated using already established methodologies for complex matrices to enhance the extraction process and

guarantee the fiber lifespan. Subsequently, the optimization of the cleaning process was established, defining the optimal parameters for the automated cleaning protocol and then tested to prove the advantages of this new automated method in the maintenance of the lifespan of this matrix compatible coatings for more than 100 extractions. The process was followed by the development of a DI-SPME methodology for the identification and quantitation of contaminants in this fatty matrix using the already established automated cleaning protocol. Satisfactory figures of merit were obtained from the matrix selected with limits of quantitation for all compounds at ranging between 0.03 and 0.1 $\mu\text{g/g}$.

Additionally, in the final chapter, the applicability of a new gas generating vial with a new solid support is presented as an alternative and more reliable source for a standard gas vial system in high-throughput SPME analysis; the new vial can stand over 300 extractions consecutively with less than 5% of depletion over time/use.

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Table of Contents

AUTHOR'S DECLARATION	II
ABSTRACT	III
ACKNOWLEDGEMENTS	V
LIST OF FIGURES	IX
LIST OF TABLES	XI
LIST OF ABBREVIATIONS	XII
CHAPTER 1. INTRODUCTION	1
1.1. Food analysis and sample preparation	1
1.2. Solid phase microextraction in food analysis	3
1.2.1. SPME Principle	3
1.2.2. SPME in food analysis.	7
1.2.3. Development of matrix compatible coatings in SPME	9
1.2.4. Automated DI-SPME	14
CHAPTER 2. DEVELOPMENT AND OPTIMIZATION OF A CLEANING PROTOCOL FOR FATTY MATRICES USING THE CTC PAL3 AUTOSAMPLER SYSTEM.	17
2.1. Introduction	17
2.2. Experimental	18
2.2.1 Chemicals and materials	18
2.2.2. SPME procedure	19
2.2.3. GC/MS equipment and analysis conditions	20
2.2.4. Cleaning station development	21

2.2.5. Statistical analysis	22
2.3. Results and Discussion	22
2.3.1 Evaluation of the cleaning process using FWM.	22
2.3.2. Different strategies for the cleaning of the fiber in the avocado extraction	26
2.4. Conclusions	37
CHAPTER 3. ANALYSIS OF DIFFERENT TYPES OF CONTAMINANTS IN AVOCADO USING SOLID-PHASE MICROEXTRACTION (SPME) COUPLED WITH GAS CHROMATOGRAPHY MASS SPECTROMETRY (GC-MS)	38
3.1. Introduction	38
3.2. Experimental	39
3.2.1 Chemicals and materials	39
3.2.2. SPME procedure	40
3.2.3. GC/MS equipment and analysis conditions	41
3.2.4. Statistical analysis	41
3.3. Results and Discussion	41
3.3.1. Evaluation of DI-SPME methodology for quantitative analysis in avocado puree.	41
3.4 Conclusions	51
CHAPTER 4. NEW TYPE OF GAS GENERATING VIAL STANDARD FOR SPME HIGH-THROUGHPUT ANALYSIS USING THIN FILM MEMBRANE.	52
4.1. Introduction	52
4.2. Experimental	53
4.2.1 Chemicals and materials	53
4.2.2. SPME procedure	54
4.2.3. GC/MS equipment and analysis conditions	54
4.2.4. Statistical analysis	55

4.3. Results and Discussion	55
4.3.1. Evaluation of the vial reusability	55
4.4. Conclusions	58
REFERENCES	60

List of Figures

Figure 1.1. SPME sample preparation and analyte absorption/adsorption time profile.	3
Figure 1.2. Solid phase microextraction modes. ¹²	6
Figure 1.3. Various SPME configurations: (A) fiber; (B) in-tube; (C) thin film; (D) magnetic; (E) in-tip; (F) stir bar.....	7
Figure 1.4. Schematic of the analyte extraction process from a sample containing a binding component.....	9
Figure 1.5. Coating selection guide.	11
Figure 1.6. Picture of the CTC PAL3 Autosampler.....	14
Figure 1.7. Fast Wash Module (FWM).....	15
Figure 2.1. Microscopic images of an SPME overcoated PDMS fiber after extractions in avocado matrix using ultra-pure water as a cleaning solution.	23
Figure 2.2. Preliminary results of four contaminants in avocado puree.	25
Figure 2.3. Microscopic pictures of a PDMS/DVB overcoated fiber before and after extraction in avocado using the CTC PAL3 Autosampler System with FWM..	25
Figure 2.4. FWM photographs showing the holes within the liners.	26
Figure 2.5. Microscopic pictures of a PDMS/DVB overcoated fiber used in avocado extraction.	27
Figure 2.6. Contaminant extraction reproducibility in avocado after 90 extraction cycles.	29
Figure 2.7. Extractions using a PDMS/DVB overcoated fiber performed in QC solutions comprised of pesticides spiked in ultra-pure water (3 ppm) in order to evaluate fiber performance before and after avocado extraction.	30
Figure 2.8. Response surface area plots for 3 of the 5 pesticides used in the avocado extraction. The pesticides shown range from a high to a low Log P.	34
Figure 2.9. Contaminant extraction reproducibility in avocado puree after 110 extractions with new speeds for the incubation/extraction step and cleaning process (rinsing and washing).	35
Figure 2.10. Microscopic pictures of a PDMS/DVB overcoated fiber used in over 110 consecutive extractions from avocado puree..	36
Figure 2.11. Instrumental QCs for the consecutive extractions from avocado puree.....	36

Figure 3.1. Series of 25 consecutive extractions from avocado puree using a PDMS overcoated fiber for five contaminants.....	43
Figure 3.2. Series of 25 consecutive extractions from avocado puree using a PDMS overcoated fiber for four contaminants.	44
Figure 3.3. Microscopic images of a PDMS overcoated fiber.....	45
Figure 3.4. Microscopic images of a PDMS overcoated fiber.	46
Figure 3.5. Comparison of extraction efficiency using different dilution solutions in avocado puree with a PDMS overcoated fiber (n = 5 samples).....	47
Figure 3.6. Matrix-match calibration curves of pesticides of 4 contaminants for quantitative analysis in avocado puree.	48
Figure 3.7. Matrix-match calibration curves of 5 pesticide contaminants for quantitative analysis in avocado puree.	49
Figure 4.1. Gas generating vial: silicon diffusion pump oil and PS/DVB particles (Left) and carbon fiber mesh coated with the PDMS/HLB (Right).....	56
Figure 4.2. Durability of the new gas generator vial using thin film membranes.	57
Figure 4.3. Quality control of the PDMS/CAR/DVB fiber after every 25 extractions of the new gas generating vial.	58

List of Tables

Table 1.1. Commercially Available SPME fiber coatings.	10?
Table 2.1. Pesticides extracted from avocado samples using the CTC PAL 3 Autosampler System.	20
Table 2.2. Fast Wash Module Parameters.....	23
Table 2.3. RSD values in the optimization of the agitation speed in avocado extraction.....	31
Table 2.4. RSD values in the optimization of the agitation speed in avocado extraction.....	32
Table 2.5. Experimental design randomized run log for the extraction of 5 pesticides from avocado puree.....	33
Table 3.1. List of analytes used for quantitation in avocado extraction using the CTC PAL 3 Autosampler System.	42
Table 3.2. Figures of merit for validation of the DI-SPME protocol in avocado pure using GC/MS.	50
Table 4.1. McReynolds standard and physicochemical properties.	56
Table 4.2. Coefficients of variation and depletion percentages after 300 extraction/injection cycles for the new gas generating vial.	57

List of Abbreviations

CAR	Carboxen
DI	Direct Immersion
DVB	Divinylbenzene
FID	Flame Ionization Detector
FWM	Fast Wash Module
GC	Gas Chromatography
HLB	Hydrophilic Lipophilic Balance
HS	Headspace
IS	Internal Standard
LC	Liquid Chromatography
LLE	Liquid-Liquid Extraction
LOD	Limit of Detection
LOQ	Limit of Quantitation
MS	Mass Spectrometry
PA	Polyacrylate
PDMS	Polydimethylsiloxane
PEG	Polyethylene glycol
QuEChERS	Quick, Easy, Cheap, Effective, Rugged and Safe
RSD	Relative Standard Deviation
SIM	Selected Ion Monitoring
SPE	Solid Phase Extraction
SPME	Solid Phase Microextraction

Chapter 1. Introduction

1.1. Food analysis and sample preparation

Food analysis constitutes one of the most noteworthy and challenging disciplines in analytical science. Globalization of the food trade has increased concerns regarding the authentication and safety of food commodities (e.g., raw food, packing materials, and final processed products),¹ propelling the adoption of increasingly more stringent international laws, policies, and standards meant to control and prevent food contamination and fraud, as well as to regulate food trade.² Consequently, the food analysis field has seen a large increase in the development of analytical methods for determination of aspects of quality and safety of fresh and processed foodstuffs, such as: authenticity, flavor, nutritional value, adulteration, and contamination.

As the safety of foodstuffs plays a large role in determining their acceptability for human consumption, the analysis of pesticide residues, which are a known health risk to humans when present in foods at high enough concentrations, has been a priority in the field for many years. Currently, more than 1000 active substances belonging to many different chemical classes are used worldwide as pesticides, having as the only common characteristic among them their potential effectiveness against pests. As such, analysis of pesticides presents a large challenge to analysts due to the wide range of analytes with different physicochemical properties to be considered.³⁻⁸

As a category of analysis, 'food' encompasses a diverse group of complex matrices characterized by widely heterogeneous compositions. Originating from either animal or vegetal sources, food matrices may contain widely varying amounts and types of nutrient and non-nutrient components, such as: proteins, fatty acids, carbohydrates, vitamins, salts, minerals, etc,⁴ posing significant challenges for analysis. Together with increased environmental concerns, which have given rise to an increased demand for more environmentally friendly methods of analysis, the challenges in food analysis stemming from the diversity of such matrices have prompted analysts to seek greener, faster, more precise and accurate methods and instrumentation to ensure the safety, quality, authenticity, and traceability of food.^{2,4,9} The determination of trace level analytes in complex food matrices often requires extensive sample preparation protocols prior the analysis.

To this end, an increased number of steps used in an analytical method, such as sample clean up and pre-concentration steps, often leads to the propagation of error in the results. Traditionally, conventional methods for determination of pesticides in food, such as liquid-liquid extraction (LLE) and solid phase extraction (SPE), among other techniques, have generally involved a laborious sample preparation process. However, recent advancements in the field have introduced simpler, faster, and greener methods for analysis of complex food samples, shifting the methodology from arduous and environmentally unfriendly methods to simpler processes covering a broader range of analytes. One such method, first introduced by Anastassiades *et al.* as a simpler sample preparation approach for multiresidue analysis of pesticides in food,¹⁰ is the QuEChERS procedure, which stands for Quick, Easy, Cheap, Effective, Rugged and Safe.

The QuEChERS extraction procedure is based on liquid-liquid extraction with acetonitrile, ethyl acetate, or acetone, and partitioning with magnesium sulphate or its combination with other salts, followed by a clean-up step (or steps) with dispersive SPE. This method effectively covers a wide scope of analytes, and therefore, is being extensively applied in multiresidue analysis of pesticides in fruits and vegetables, and most recently, to food commodities of animal origin. One of the main drawbacks of this method entails the relatively low pre-concentration capability per sample, requiring that final extracts be concentrated so as to achieve acceptable limits of quantification (LOQ). In addition, QuEChERS, as a multistep method, is burdened by challenges related to automation of the analytical workflow, as combining the sample preparation and instrument introduction steps is not easily accomplished for this method. As an alternative to QuEChERS, solid phase microextraction (SPME) is presented as a promising solvent-free technique that offers a fast, green, and easy sampling-sample preparation process capable of overcoming typical limitations associated with analysis of complex food matrices.

1.2. Solid phase microextraction in food analysis

1.2.1. SPME Principle

Solid Phase Microextraction (SPME) is a well-known and accepted sample preparation technique developed by Pawliszyn *et al.* in the 1990s.¹¹ SPME is a non-exhaustive extraction technique that integrates various workflow steps, such as sampling, isolation of analytes from interfering compounds, and the enrichment/pre-concentration of analytes of interest. In this technique, a solid support coated with a small amount of extracting phase (coating) is exposed to the sample matrix for a defined period of time (Figure 1.1A).^{11,12} Analytes diffuse from the sample matrix to the coating, where analytes are either absorbed within the extracting polymer or adsorbed onto its surface. Figure 1.1B shows the time profile of the analyte sorption onto the SPME coating.

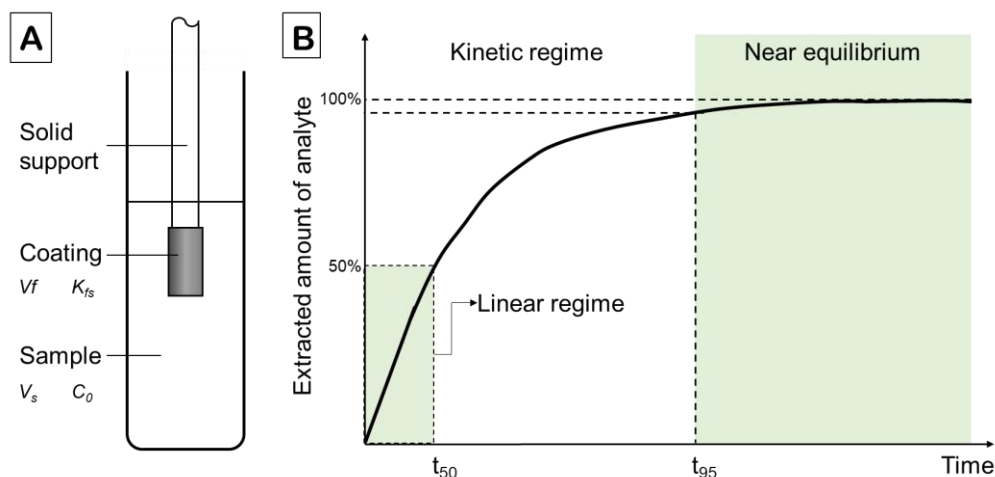


Figure 1.1. SPME sample preparation and analyte absorption/adsorption time profile. SPME sample preparation and analyte absorption/adsorption time profile. SPME sample preparation and analyte absorption/adsorption time profile. SPME sample preparation and analyte absorption/adsorption time profile. (A) Sample preparation scheme for SPME; where V_f is the fiber coating volume, K_{fs} is the fiber/sample distribution coefficient, V_s is the sample volume, and C_0 is the initial concentration of analyte in the sample. (B) Typical time profile of analyte extracted in

SPME. t_{50} , time required for extraction of half maximum analyte; t_{95} , time required for extraction of 95% maximum analyte.¹²

Typically, the microextraction process is considered complete when analyte concentration equilibrium is reached between the sample matrix and the extraction phase; according to the law of mass conservation, equilibrium conditions can be described by Equation 1

$$C_0 V_s = C_s^\infty V_s + C_f^\infty V_f \quad (1)$$

Where C_f^∞ and C_s^∞ are the equilibrium concentrations in the fiber coating and the sample, respectively. K_{fs} , the distribution coefficient of analytes between the fiber coating and sample matrix, can be described by Equation 2:

$$K_{fs} = \frac{C_f^\infty}{C_s^\infty} \quad (2)$$

Combining and rearranging Equation 1 and Equation 2, the number of moles of analyte n extracted by the coating once system equilibrium is reached, within the limits of experimental errors, can be calculated from Equation 3:¹²

$$n = C_f^\infty V_f = C_0 \frac{K_{fs} V_f V_s}{K_{fs} V_f + V_s} \quad (3)$$

Where n is the number of moles extracted by the coating, K_{fs} is the phase/sample matrix distribution constant, V_f is the fiber coating volume, V_s is the sample volume, and C_0 is the initial concentration of analyte in the sample. Equation 3 thus describes the analytical basis for quantification using SPME, where the amount of analyte extracted onto the coating, n , is linearly proportional to the concentration of said analyte in sample C_0 .^{12,13} It should be noted that Equation 3 is only valid for applications involving liquid polymeric coatings; in cases where a solid polymeric coating is employed as extraction phase, the equation should consider the surface area on the fiber instead of the volume of the fiber.

This non-exhaustive extraction method can be carried out under two regimes of extraction, equilibrium and pre-equilibrium. Under the equilibrium regime, SPME is carried out by exposing the extraction phase to the sample matrix until enough time has elapsed so that analyte concentration equilibrium has been reached between phase and sample; once reached, the amount of analyte extracted to the phase will remain the same regardless of additional exposure time.¹² If not enough time is allowed for equilibration, pre-equilibrium regime, the amount of analyte extracted can be estimated with respect to the time of exposure (Figure 1.1B).¹²

As equilibrium times in SPME can be excessive, practical equilibrium is assumed to be achieved at a time point when 95% of the equilibrium amount of analyte is extracted from the sample (Figure 1.1B). One of the advantages of carrying out extractions close to t_{95} involves the prospect of incurring a lower relative error with respect to the extracted amount of analyte as compared to that which can be incurred by sampling while in the kinetic regime. Extractions performed in the kinetic regime require a precisely timed analysis to minimize the relative error on the amount of analyte extracted.⁴ Nonetheless, extractions performed in the kinetic regime require shorter analysis times as compared to equilibrium-based extractions. As such, provided that they render sufficient method sensitivity, kinetic-regime extractions present a preferable alternative for applications that require fast throughput, as well as for applications involving extractions that require long equilibration times, as is the case for most analyses involving complex matrices. Equation 4 is the non-linear equation describing the kinetics of analyte absorption and the relationship between analyte extracted and extraction time.^{4,12}

$$n(t) = [1 - e^{(-a_e t)}] C_0 \frac{K_{fs} V_f V_s}{K_{fs} V_f + V_s} \quad (4)$$

Where n is the number of moles extracted; t is the extraction time; and a_e , is an extraction rate constant that is dependent on the extracting phase, sample volume, mass transfer rate, partition coefficients, and the surface area of the extracting phase. This rate constant refers to how fast equilibrium is reached.

SPME can be performed via different modes of extraction, the most common being headspace SPME (HS-SPME), and direct immersion SPME (DI-SPME) (Figure 1.2). Sampling mode is generally selected based on the type of sample matrix under study and the nature of the analytes of interest. In headspace mode, the analytes under study must be volatile enough so as to distribute

between the sample matrix and the vial headspace, where the SPME device is exposed for extraction. The advantage of using this sampling mode is that it circumvents typical fiber damage associated with DI-SPME (e.g., fiber fouling, mechanical damage).^{9,12}

In direct immersion mode, the fiber is directly exposed to the sample matrix; thus, the analytes are directly transported from the sample and onto the coating. Agitation plays a critical role in SPME processes, as it can aid in the attainment of shorter equilibrium times, or, in cases where extraction is carried out under the pre-equilibrium regime, it can accelerate the uptake rate of analytes. In most DI-SPME applications, use of sample agitation can greatly aid the extraction process, as analytes need to be transferred and diffused from the bulk of the sample and onto the coating. In headspace mode, where the natural convection of air is frequently great enough for extraction to occur, agitation can help shorten extraction times. Additionally, some modifications to the sample matrix, such as changes to pH and ionic strength, can enhance the extraction process, in both extraction modes.^{9,12}

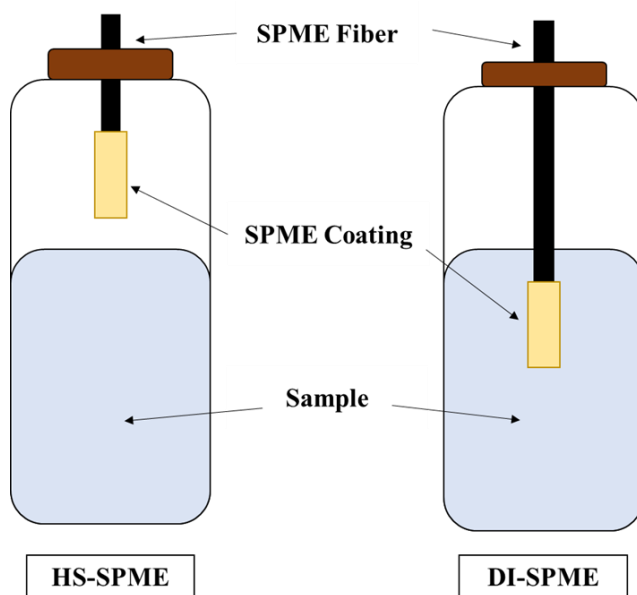


Figure 1.2. Solid phase microextraction modes.¹²

Through the years, the applicability of SPME has been expanded to different fields of study, introducing a wide-ranging variety of new applications as well as novel geometries and configurations of SPME devices to fulfil diverse analytical needs (Figure 1.3). The simplicity of the first design of the fused silica fiber,¹¹ which enabled easy coupling of the technique to gas chromatography (GC) instrumentation, played a vital role in the early automation of SPME in GC applications. Over the years, autosamplers have evolved to enable the performance of completely automated SPME-GC workflows, even affording automation of previously manual steps such as fiber exchange.^{12,14,15}

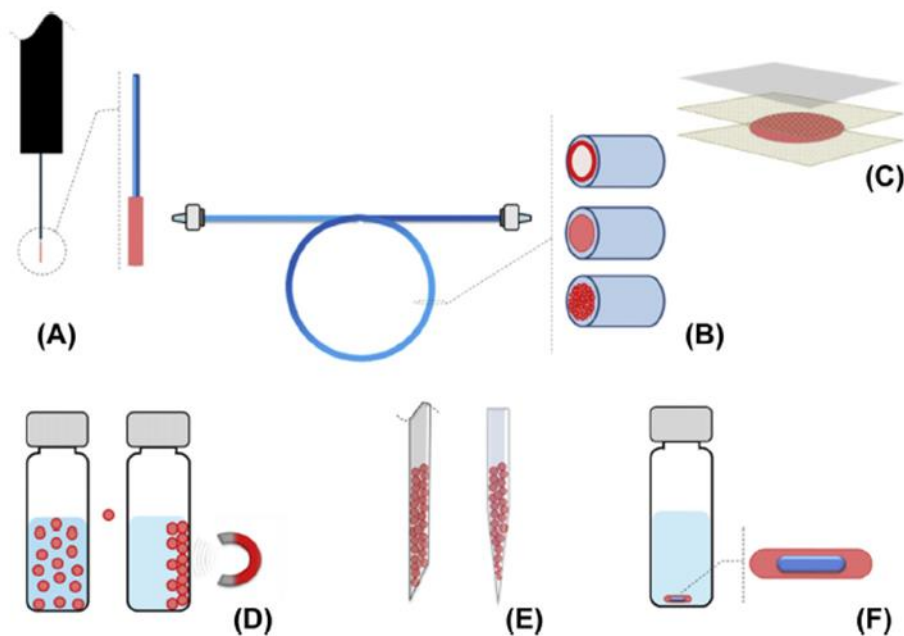


Figure 1.3. Various SPME configurations: (A) fiber; (B) in-tube; (C) thin film; (D) magnetic; (E) in-tip; (F) stir bar.¹²

1.2.2. SPME in food analysis.

In food analysis, the complexity of the matrix under study represents one of the most challenging aspects to consider with respect to method development. Since its introduction, the applicability

of SPME towards food analysis has expanded into different food areas.¹² Within this context, applications directed at the analysis of fragrance and odor of food commodities have evidenced great expansion in recent years, as aroma and flavor represent important factors in overall food quality and reception, being directly linked with consumer approval and the perception of food quality.^{16,17} As previously mentioned, SPME offers two extractive modes (HS- and DI-SPME) for analysis in complex matrices, enabling a wide range of applications related to the analysis of various fragrance and odor components, as well as determinations of contaminants present in food samples.^{12,16} In this respect, headspace mode (HS-SPME) has been widely employed for the determination of volatile compounds such as aroma and flavor compounds.^{5,16} Over the years, reported applications of HS-SPME have included aroma profiling,^{5,12,17–20} origin,^{5,12,17,21,22} and contamination,^{5,12,17,23} among others.^{5,12} However, the principal limitation of this sampling mode lies in the poor “balanced coverage” of analytes that it affords, particularly its low applicability towards determinations of less volatile compounds. Such a limitation is inherent to headspace applications, as such techniques do not enable detection and quantification of non-volatile compounds, owing to the physicochemical properties of such compounds and the interactions among them and the sample matrix.¹⁶ Moreover, HS-SPME fibers perform poorly in complex matrices and have reduced lifespans when used for DI extractions. Conversely, the direct immersion mode (DI-SPME) of SPME enables quantification of non-volatile compounds, enabling “balanced coverage” of analytes, provided a suitable phase is selected for extraction.^{5,12,17,24–27} Once exposed directly to the sample media, the extraction efficiency of SPME for polar, semivolatile, and non-volatile compounds increases drastically, “as the diffusion coefficients through the matrix, that define the mass transfer properties of the extraction, are similar for all the small molecules present in the system; therefore, a more comprehensive analyte coverage is obtained.”^{16,28} As a result, the “balanced coverage” feature of DI-SPME constitutes a useful tool for new approaches in food analysis and food metabolomics.⁵

In this regard, the balanced coverage capability of SPME is based to its ability to extract compounds via free concentration;¹⁶ which requires consideration of kinetics of binding equilibria for complex matrices (Figure 1.4).^{12,16,29} As shown in Figure 1.4, analyte extraction is dependent on the distribution constant between the coating and the sample, K_{fs} , and its binding constant with the matrix binding components, K_a . Here, the binding constant, K_a represents the relationship that exists between the two binding reaction constants, the forward k_f and backward k_r .²⁹ When present

in complex matrices, hydrophobic compounds are usually heavily bound to the sample matrix, and thus present in low free concentrations. However, hydrophobic compounds have high affinity for SPME phases; as a result, SPME enables high recoveries of hydrophobic compounds from complex samples. On the other hand, although polar compounds are generally present in complex matrices in high free concentrations due to their almost null binding with matrix components, such compounds generally display a very low affinity for SPME coating phases, allowing for a proportional amount of such compounds to be extracted from complex matrices. These combined effects result in the “balanced coverage” effect afforded by SPME, provided a suitable coating is employed for extraction.²⁹

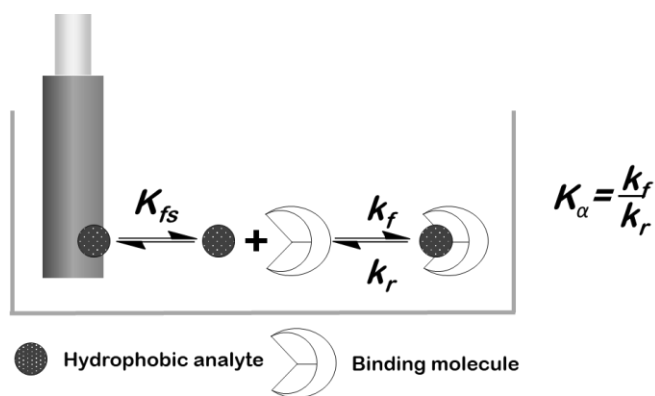


Figure 1.4. Schematic of the analyte extraction process from a sample containing a binding component.¹²

1.2.3. Development of matrix compatible coatings in SPME

The suitability of SPME for a given application is critically dependent on the availability and selection of an appropriate coating for extraction.¹² Considering that the properties of the coating are responsible for the selectivity of SPME for the target analytes, the types of coatings available for SPME play a large role in its aptness for a given application. To that extent, categorization of commercially available SPME coatings should take into consideration four factors that play a role in their extractive performance: type of coating, coating thickness, polarity, and the absorption

mechanism of the coating.¹² Table 1.1¹² presents a list of commercially available SPME fibers suitable for GC applications. Although SPME enables extraction of a wide range of analytes, from volatile to non-volatile, and from polar to non-polar compounds, the selected coating determines the range of analytes that can be extracted from a given matrix. Figure 1.5 illustrates a typical coating selection process that takes into account the polarity and volatility of the analytes under study.¹²

Since the inception of SPME, the continuous development of new SPME coatings has aimed at overcoming limitations related to operation temperature, selectivity, robustness, carryover, swelling in solvents, and cost-affordability.^{5,30} Of the limitations inherent to DI-SPME for analysis of complex matrices such as food, fiber fouling has, until recently, played a large role in limiting the development of new SPME applications. Fouling is caused by irreversible attachment of macromolecules present in complex matrices onto the coating surface. The fouling process leads not only to a substantial decrease in fiber lifetime, turning fibers unusable for future extractions, such a process can also change the extraction properties of the coating, which will affect extraction capabilities of the coating and possibly yield irreproducible results.³¹⁻³³

Table 1.1. Commercially Available SPME fiber coatings.¹²

Type of Coating	Extraction Mechanism	Polarity
7 μm PDMS	Absorbent	Non-polar
30 μm PDMS	Absorbent	Non-polar
100 μm PDMS	Absorbent	Non-polar
85 μm PA	Absorbent	Polar
60 μm PEG (Carbowax)	Absorbent	Polar
15 μm Carbowax Z-PDMS	Adsorbent	Bipolar
65 μm PDMS-DVB	Adsorbent	Bipolar
55 μm /30 μm DVB/Carboxen-PDMS	Adsorbent	Bipolar
85 μm Carboxen-PDMS	Adsorbent	Bipolar

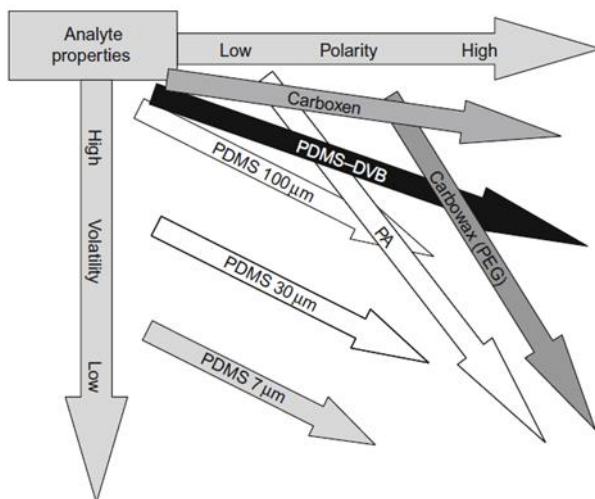


Figure 1.5. Coating selection guide.¹²

Given the complex nature of food matrices, implementation of the direct immersion (DI-SPME) approach can be difficult to achieve without coating damage. Thus, DI-SPME methods for analysis of complex food matrices may need to include some sample pretreatment or cleanup steps prior to extraction to protect the coating from mechanical damage and avoid fouling of the extraction phase.³¹ Aiming to increase the applications of SPME in food analysis and bioanalytical and clinical analysis, notable recent developments have included the introduction of the PDMS overcoated³¹ fiber and the biocompatible SPME^{34,35} coating, which aim at reducing the incidence of fiber fouling that has hindered further development of DI-SPME methods for complex matrices to date.

With respect to GC applications, Souza-Silva *et al.*^{31,32,36} recently introduced the overcoated fiber as a matrix-compatible fiber suitable for DI-SPME applications involving complex food matrices, demonstrating its capability to largely minimize damage on the coating surface via the addition of a layer of PDMS polymer to an already commercially available PDMS/DVB fiber. For this work, selection of a PDMS layer was based on the well-established suitability of PDMS as an SPME coating for pesticide analysis in food matrices.³⁷ PDMS forms a nonporous liquid coating with a homogeneous and smooth surface; these properties, alongside its robustness in direct immersion approaches, dramatically decrease the irreversible fouling caused by matrix components as compared to that which is incurred by solid coatings (e.g., PDMS/DVB coating). In addition to decreasing the amount of matrix constituents attached to the coating, the PDMS layer also enabled

easy cleaning of fibers; in cases where the surface of PDMS incurred attachment of matrix components, physical cleaning of the polymer surface was shown to sufficiently remove coating surface attachments, enabling continuous use of coatings. As a proof of concept, the overcoated PDMS/DVB fiber was used in DI-SPME-GC analysis of pesticides in diluted fruit and vegetable matrices.³¹ In comparison to commercially available PDMS/DVB fibers, the overcoated PDMS/DVB fiber afforded an increase in retention capacity, higher distribution coefficients, smaller diffusion coefficients, and higher selectivity for the analytes under study.³¹ Preparation of the aforementioned fiber entailed the immersion of commercially available PDMS/DVB fibers in a PDMS solution at a speed of 0.5 mm/s via the dip-coating approach,³⁸ after which the fiber passed through a micropipette tip of about 350 μm diameter aperture to ensure that a thinner layer was formed, and that any excess polymer was removed. The fiber was cured at 50 °C under a nitrogen (N_2) flow for 12 hours. The coating optimization process included physical evaluations of the coating under the microscope and subsequent optimization of the dip coating process, concluding that two layers of the liquid polymer PDMS enabled optimum surface coverage of the PDMS/DVB fibers. The overcoated fiber was applied towards the extraction of triazole pesticides from water and grape pulp in an evaluation of its extraction performance and lifetime.³¹ While the extraction capabilities of the PDMS/DVB/PDMS fibers toward the selected analytes from water samples were proven to be similar to those exhibited by the original commercial PDMS/DVB coating, the attained results for complex matrix investigations revealed that the modified PDMS/DVB/PDMS coating provided enhanced robustness. This was shown by investigations in matrices such as whole grape pulp when compared to the original commercially available PDMS/DVB, enabling 130 consecutive uses in comparison to the 20 uses achieved by the commercial fiber. One of the most notable practices introduced in this work is the optimization of cleaning steps prior to desorption and post-desorption of the new fiber. In this work, deionized water was used as solvent to “wash” the fiber after exposure to the complex food matrix, with the first “wash” lasting 50 seconds. The second wash, carried out after thermal desorption of the overcoated fiber, was applied for 2.5 minutes, using the same solvent. The introduction of these washing steps was intended to help extend the life of the fiber; in addition, the fiber was also cleaned with Kim Wipe tissue in cases where matrix constituents were not sufficiently removed with deionized water.³²

In other work by Souza-Silva *et al.* that included employment of the overcoated PDMS/DVB/PDMS fiber,³² the applicability of the fiber for determination of pesticides in grapes and strawberries was compared with that of the QuEChERS method. The cleaning process of the fiber was similar to the first work carried out by Souza-Silva *et al.*,³¹ where the fiber was “washed” prior to thermal desorption with deionized water as washing solvent. In both studies, the performance of the new PDMS overcoated fiber was clearly superior in DI-SPME analysis of complex food matrices. However, no further details were given regarding washing steps after thermal desorption, including any particulars pertaining to the use of Kim Wipes for manual cleaning of the fiber. It is important to mention that after the multiple trials and this detail work using the PDMS overcoated fibers, they were and are commercially available now for application regarding complex matrices.

Risticovic *et al.*^{24,25} used PDMS overcoated fibers for analysis of apple samples, developing a method to study different types of metabolites in apples in *ex vivo* and *in vivo* conditions. This whole study involved three steps for the evaluation of the extractive performance of commercially available SPME fibers in terms of extraction selectivity, sensitivity, and desorption efficiency. In the first step, aqueous standards as well as homogenized apple samples were spiked with representative volatile and semivolatile metabolites, for extraction employing HS-SPME mode. This preliminary study established the use of DVB/CAR/PDMS fibers for the following analysis. In the second step, the selected fiber was used for real apple samples for a metabolite profiling approach. In the third step, the DVB/CAR/PDMS coating, selected on the basis of optimum metabolite coverage and extraction sensitivity, was employed for *ex vivo* and *in vivo* sampling assays for determination of volatile and semivolatile metabolites in apples. The DI-SPME extraction mode was selected for sampling due to the balanced coverage it affords. This is the first report illustrating the implementation of an *in vivo* DI-SPME assay for non-invasive determination of endogenous fruit metabolites, whose profiles and contents are highly correlated to a multitude of influential fruit quality traits. The cleaning process for this DI-SPME extraction mode in *ex vivo* and *in vivo* analysis of apple samples consisted of a brief immersion of the fiber in nano-pure water, followed by manual cleaning of the fiber with Kim Wipes after fiber exposure to the sample, as well as prior to thermal desorption the fibers.

1.2.4. Automated DI-SPME

Based on the positive results of previous work realized by De Grazia *et al.*³⁹ as proof of concept for the use of DI-SPME in a fatty matrix, like avocado, for more than 100 extractions using a recent developed and now commercially available PDMS overcoated fiber, the interest in accomplish complete automation of the workflow for multiresidue analysis of contaminants or residual pesticides content in these type of complex matrices is possible and therefore must be investigated.

The use of new technologies is important to the development of analytical techniques; automation of new SPME methodologies can be tested and enhanced using a CTC PAL3 System Autosampler (Figure 1.6) that offers new tools to strengthen the automated SPME process.

Aiming to provide greater flexibility and security for routine and research applications, the third generation of this well-known PAL System (Prep and Load System) presents several advantages to the user. The autosampler offers a unique park station, with three automated tools for advanced sample preparation: an SPME tool, a headspace tool, and a liquid syringe tool. It also offers the typical module for incubation/agitation, normal and large static wash stations for syringe cleaning, and an expansion of the number of tray holders, offering the use of a large range of vial sizes. Two new modules are also presented to enhance the applicability of the autosampler: the vortex mixer and the fast wash module (FWM). These modules are intended to expand the range of applications enabled by the instrument by adding versatility to the automated system.⁴⁰



Figure 1.6. Picture of the CTC PAL3 Autosampler.

As mentioned above, the fast wash module (Figure 1.7) is one of the new features that could be employed toward enhanced automation of the aforementioned SPME methods. Initially designed for cleaning of liquid syringes in the system, it can be used as an interesting station for rapid cleaning of SPME fibers after exposure to complex matrices, where mechanical attachments may occur.

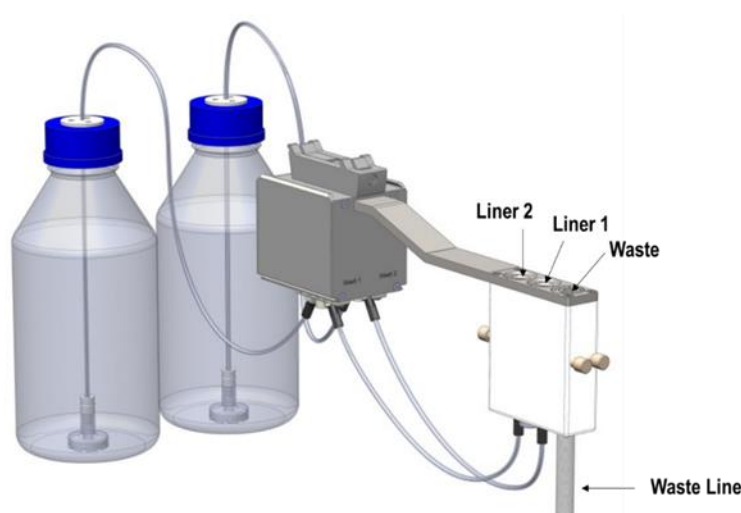


Figure 1.7. Fast Wash Module (FWM). Figure adapted from PAL3 System User Manual.⁴⁰

The FWM has two micro pumps, each attached to a different liner that actively carries solvent from a container to the cleaning station after insertion of the syringe or SPME fiber, effectively enabling control over the flow of solvent being delivered. Once delivered to the station, the solvent is then transported to the waste outlet, enabling the performance of multiple cleaning steps without concerns over waste accumulation, thus fulfilling its proposed use as a wash station, and not merely a solvent storage unit.⁴⁰

The features afforded by the autosampler allows the user to perform different automated approaches via the different modules available on the instrument, which can be controlled manually, or through employment of the CTC software. While multiple parameters can be evaluated for the different modules (agitator/incubator, vortex mixer), the completed evaluation of

multiple parameters that can be modified in the different modules of this new autosampler with the primary focus of enhancing the analysis of the aforementioned complex food matrices by improving the lifespan of the fiber.

Chapter 2. Development and optimization of a cleaning protocol for fatty matrices using the CTC PAL3 Autosampler System.

2.1. Introduction

Recent developments in matrix-compatible coatings have increased applications for DI-SPME in food, biological, and environmental analysis. One of the most critical steps in the DI-SPME protocol is the cleaning of the SPME device, which can be done in two ways: by using a washing solution at the end of the SPME extraction and prior to thermal or solvent desorption, or by using a manual cleaning process that usually involves a break in the automated SPME protocol.

It is important to establish a cleaning protocol that will help to extend the lifetime of the fiber, as this will ensure that the coating maintains consistent performance throughout the extraction process and between extraction cycles, thus ensuring reproducible results. To this end, almost all applications involving PDMS overcoated fibers have thus far been tested within an automated SPME workflow, usually using a CTC Combi-PAL autosampler. However, the cleaning process adopted for these workflows is principally limited by the need to clean the fibers manually with Kim Wipes between extractive cycles, as evidenced in the above-mentioned studies of grape and apple matrices. In previous works involving the use of fatty matrices, such as De Grazia *et al.*, the application of organic solvents during the cleaning step created significant limitations for the overall process due to multi-residue determinations. The presence of an organic solvent in the rinsing solution prior to thermal desorption causes the solution to act “as an additional phase that can actually compete with the SPME coating for the partition of analytes by inducing their back-extraction from the coating into the rinsing solution”.^{5,12,39} This in turn can lead to analyte loss in multi-residue pesticide determination, as well as a lack of method reproducibility. In view of this, it is necessary to continue to develop methods that are able to provide more comprehensive and accurate results, while also enabling complete workflow automation. In addition to increasing analysis throughput and decreasing the labour required to implement it, workflow automation also helps to prevent errors stemming from manual processes associated with fiber cleaning.

Consequently, it is worth exploring how new technologies can be integrated into automated systems in order to enhance existing sample preparation protocols. SPME is one such sample preparation protocol that has enabled the reduction or complete elimination of organic solvent use during sampling/sample preparation. However, the growing use of DI-SPME protocols in multiple fields and for a greater range of applications has led to an increase in the use of organic solvents during the cleaning process due to the complexity of the analyzed matrices. In response, CTC Analytics introduced a Fast Wash Module (FWM) in the new version of their automated system, offering users a powerful new tool that can be used to improve the cleaning process. The FWM was introduced with two principal objectives in mind: to reduce and/or eliminate the use of organic solvents during the cleaning step of DI-SPME analyses of fatty matrices (avocado analysis), and to enhance automation by guaranteeing the use of fresh solution during each cleaning step thanks to the module's solvent reservoirs.

2.2. Experimental

2.2.1 Chemicals and materials

All chemicals—namely nitrobenzene, trifluralin, 4-phenylphenol, p,p'-dichlorodiphenyldichloroethylene (p,p'-DDE), and thiabendazole—were supplied by Sigma Aldrich (St. Louis, MO, USA) (

Table 2.1), while HPLC-grade methanol, acetonitrile, and acetone were purchased from Fisher Scientific (Bartlesville, OK, USA). Purified water was obtained using Milli-Q systems (Waterloo, ON, Canada). A stock solution was prepared in methanol using concentrations of each chemical at 800 ppm, and then stored in a refrigerator at $-30\text{ }^{\circ}\text{C}$ until use. Different dilutions of this solution were prepared as needed in accordance with the concentration levels required for the type of matrix being used (water or avocado). Polydimethylsiloxane (PDMS)/Divinylbenzene (DVB) (PDMS/DVB) overcoated fibers (65 μm coating thickness + 10 μm PDMS layer) and PDMS/DVB fibers (65 μm coating thickness) were purchased from Supelco (Bellefonte, PA, USA). Samples

of organic avocado fruits (*Persea americana* ‘Hass’) were purchased from local grocery stores, with the avocado pulp being cut into pieces and homogenized using an electric blender prior to SPME extraction.

2.2.2. SPME procedure

The spiking procedure and quality control (QC) used during the SPME procedure were conducted according to the conditions outlined by De Grazia *et al.*³⁹ For the QC analyses, solutions containing ~3 ppm of each chemical pollutant were created by spiking 25 μL of stock solution (800 ppm) in 7 mL of deionized water in order to dissolve the analytes. The SPME fiber was then immersed in the solution for 40 min at 35 °C under agitation conditions (425 rpm).

For the avocado sample analyses, 20 g of homogenized avocado pulp was weighed in a 40 mL amber vial, combined with 400 μL of a standard solution of 800 mg/L, and stirred at 1500 rpm overnight (10-12 hours) using a MultiTube Vortexer (Fisher Scientific, Waltham, MA, USA). After stirring, 2.1 g of the spiked fruit pulp was transferred to a 10 mL amber vial and diluted with 4.9 mL of deionized water (dilution level 30:70%), producing matrices with final analytes concentrations of 4.8 $\mu\text{g/g}$. Initially, SPME extraction was performed by first incubating the sample for 1 min at 35 °C, and then directly exposing the fiber to the sample for 40 min at 35 °C under agitation at 425 rpm. After sampling, the fiber underwent 5 s of pre-desorption rinsing in acetone/water (9:1, v/v) at 425 rpm. Desorption was conducted in the injection port for 5 min at 270 °C in splitless mode, and was followed by a 30 s post-desorption washing step in pure acetone at 425 rpm. However, since the objective was to evaluate the suitability of the FWM, different trials were conducted using the module’s two different settings and compared to the above approach.

The avocado sample analyses were carried out in order to ensure that after every 10 extractions, a quality control (QC) analysis was performed by conducting SPME extractions from water solutions containing pollutants at the same levels as in the real samples. Each QC analysis was conducted in triplicate using a commercial PDMS/DVB fiber specifically designated for this purpose. The experimental results were normalized according to the QC response in order to

account for instrumental response drifts, and to ensure that the evaluated responses were exclusively affected by the coating extraction efficient.⁴¹

2.2.3. GC/MS equipment and analysis conditions

An Agilent 6890-5977A GC-MS (Mississauga, ON, Canada) equipped with a DB-5 MS column (30 m, 0.25 mm I.D., 0.25 μ m film thickness) (Agilent Technologies, Santa Clara, CA, USA) was used for this research. The DI-SPME methodology's sensitivity was further enhanced via a Selected Ion Monitoring (SIM) method, which was developed using the m/z ratios and retention times of the target analytes. Helium (purity 99.999%) was selected as the carrier gas, and was fed into the column at a linear velocity of 1.5 mL/min. The oven temperature was set to 40 °C and held there for 2 min before being raised to 180 °C at a rate of 10 °C/min, and finally to 300 °C at a rate of 20 °C/min where it was held for an additional 5 min. Throughout this process, the injector was held at 270 °C, the transfer line temperature was held at 250°C, the MS source was held at 230°C, and the MS quadrupole was held at 150°C. The GC-MS instrument was controlled by a PC running Agilent Masshunter Qualitative Analysis software. Finally, this research employed a CTC PAL 3 Autosampler System equipped with a Fast Wash Module (FWM), a six-vial agitator/incubator, a vortexer, an SPME conditioning module, an SPME park station, and headspace and liquid-injection tools.

Table 2.1. Pesticides extracted from avocado samples using the CTC PAL 3 Autosampler System.

Pesticides	Molecular Weight (g/mol)	Log P (pH 7)	Retention Time (min)	m/z ratio	Boiling Point (°C)
Nitrobenzene	123.06	1.90	8.30	77	210.9
Trifluralin	335.28	5.07	15.10	306	139.5
4-Phenylphenol	170.21	3.20	16.30	170	305.0-308.0
p,p'-DDE	318.02	6.00	23.40	246	336.0

Thiabendazole	201.24	2.47	22.20	201	-
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* Analytes highlighted in blue were use only at the beginning of the trials with the FWM.

All physicochemical data was collected from NIST Chemistry WebBook, online database (<https://webbook.nist.gov/chemistry/name-ser/>, accessed on February 12, 2018).

2.2.4. Cleaning station development

One of the main goals of this research was to develop an automated approach to multi-residue pesticide analysis in avocado samples. As such, a short experiment was conducted using avocado samples in order to determine the capacity of the CTC PAL 3 System and to evaluate the FWM's performance in comparison to the method for cleaning overcoated fibers used with this matrix detailed by De Grazia *et al.*

Before executing the preliminary test of the new autosampler, it was first necessary to create an SPME method for the FWM. As discussed in Chapter 1, the FWM was not designed for use with SPME fibers, so CTC Analytics' assistance was sought in adapting the device for SPME processes, as well as in securing the integrity of the module and the autosampler within the work that was going to be performed.

CTC Analytics helpfully provided two methods for using the FWM with SPME fibers. The first method entails turning the pump on and allowing the module liner to fill. Once the liner is filled, the pump is turned off again and the fiber is exposed to the cleaning fluid for a predetermined time period. After the fiber has been cleaned, the pump is turned on again and the solvent is flushed from the liner. This procedure can be repeated as many times as the cleaning process requires (named: *Set up*). In the second approach, the pump is turned on and the fiber is exposed while the pump is working; this approach ensures that there is fluid flowing within the liner, which may enhance the cleaning process. After the cleaning period elapses, the fiber is removed and the pump is turned off again (named: *Rinse*).

These methods were tested using avocado samples that were spiked with a known concentration (800 ppm, final concentration 4.8 µg/g) of four types of pesticides (

Table 2.1, blue shadow). The traditional method for cleaning overcoated fibers includes a rinsing step wherein the fiber is rinsed with an acetone/water (9:1 v/v) solution for 5 seconds at 425 rpm before thermal desorption in the GC injector, and a washing step using pure acetone for 30 seconds after thermal desorption. The tests of the methods provided by CTC Analytics used the same rinsing/washing solutions and cleaning times as the traditional cleaning protocol.

2.2.5. Statistical analysis

Microsoft Excel v16.14 and the Tibco Statistica 13.3 basic academic bundle were used for statistical analysis.

2.3. Results and Discussion

2.3.1 Evaluation of the cleaning process using FWM.

For the initial avocado extractions, the principal parameters of the Fast Wash Module (Table 2.2) were set to their maximum values, with ultra-pure water being used as a cleaning solvent in the rinsing/washing steps for both methods provided by CTC Analytics. After each test, the fiber surface was qualitatively evaluated, with the results indicating that the lifetime of each fiber was significantly reduced after only 5 extraction cycles from avocado (Figure 2.1). Thus, on its own, ultra-pure water is not an adequate cleaning solvent for use with fatty matrices, as it is ineffective at extending the life of the SPME fiber.

Table 2.2. Fast Wash Module Parameters.

Fast Wash Module (FWM)				
Equipment	Parameters	Range of Operation	Maximum Value	Default Value
2 Micro Pumps	Flow Rate	5-60 $\mu\text{L/s}$	60 $\mu\text{L/s}$	60 $\mu\text{L/s}$
2 Rinse Liners	Liner Penetration Depth*	15-50 mm	50 mm	25 mm
	Penetration Speed	10-100 mm/s	100 mm/s	40 mm/s
	Depenetration Speed	10-100 mm/s	100 mm/s	100 mm/s
Solvent Reservoir	Volume	100-1000 mL	1000 mL	-

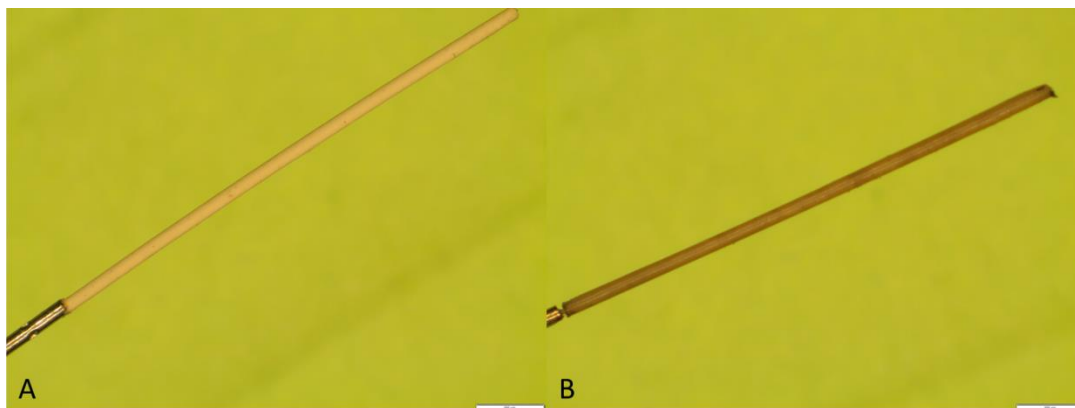


Figure 2.1. Microscopic images of an SPME overcoated PDMS fiber after extractions in avocado matrix using ultra-pure water as a cleaning solution. **A.** Overcoated PDMS fiber after thermal conditioning (0.6X magnification). **B.** Overcoated PDMS fiber after 5 extractions in avocado puree (0.6X magnification).

Conversely, promising results were obtained using solvents traditionally employed in the DI-SPME protocol, as well as via manually cleaning the fibers with Kim Wipes and pure acetone prior to changing the FWM method. However, after 20-25 sample extraction cycles using the same

SPME overcoated fiber, it was clear that this cleaning process did not successfully remove the matrix from the fiber, even though the evaluated parameters—namely, the SPME fiber exposure mode, the flow rate of the pumps attached to each liner, the depth of SPME penetration into the FWM for exposure to solvent, and solvent composition for the rinsing/cleaning process for the FWM—had been programmed to their maximum settings. Specifically, pump flow was set to the maximum level allowed by the module (60 $\mu\text{L/s}$), the fiber's depth of penetration in the FWM was also set to the maximum value (50 mm), and pure acetone was used as both a rinsing and washing solution.

Figure 2.2 lists the preliminary results for four pesticides after 30 extraction cycles using the FWM and provides a comparison of the two cleaning cycles developed for this module with the cleaning procedure reported by De Grazia *et al.* Although the RSDs for the two developed cleaning cycles were less than 40% for all the compounds over all 30 cycles, the methodology described by De Grazia *et al.* was able to produce RSDs of less than 25%. The decrease in the area counts of the compounds following either of the two developed FWM cleaning methods can be directly linked to the module's poor performance during the cleaning protocol for the overcoated PDMS fiber. This poor performance could be the result of poor pump flow rate, the position of the fiber in the FWM liner, or even the composition of the cleaning solvent. Indeed, it is possible that the use of acetone may have affected the initial rates of analyte extraction from the coating by washing them off along with the matrix attachments. To ensure that the cleaning methods were working, the fiber was visually inspected at the beginning and end of every 10 cycles for each cleaning method. In addition, the fibers were also cleaned with Kim Wipes and pure acetone to guarantee that the obtained values only referred to the cleaning method that was being tested. Figure 2.3 shows the condition of the fiber after the second cycle of 10 extractions each of the 3 cleaning methodologies (90 extractions from avocado were performed).

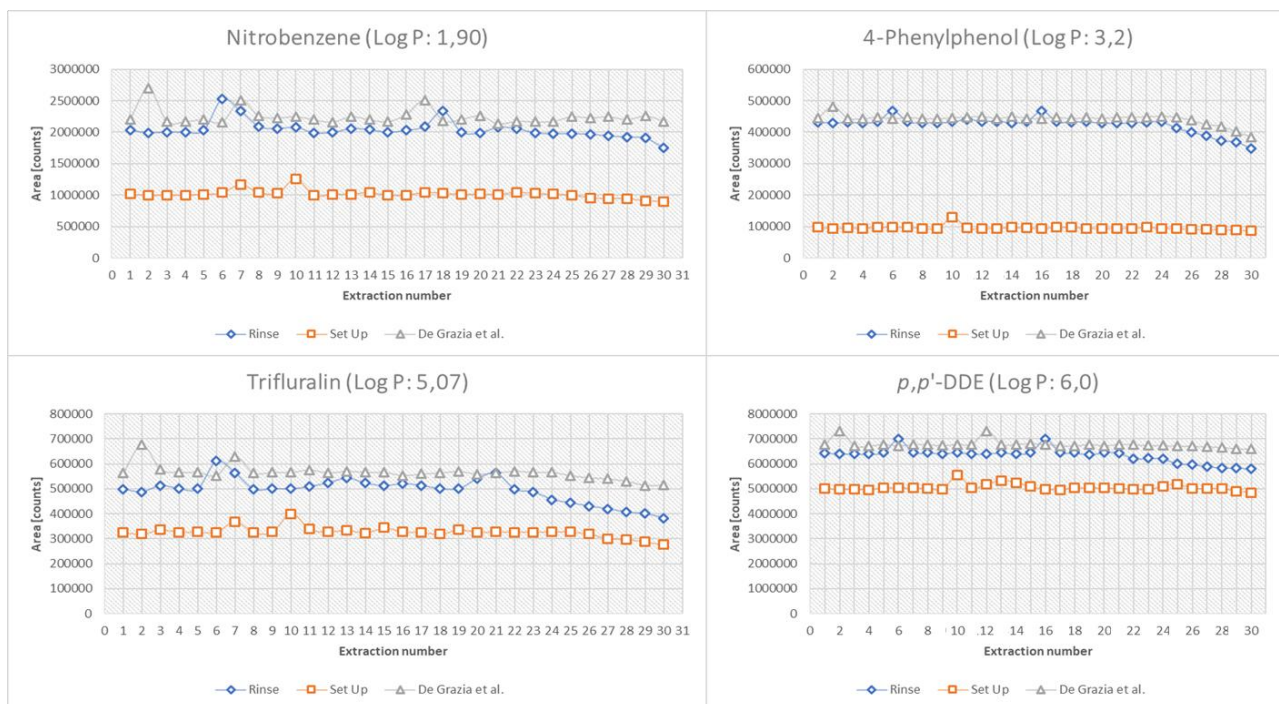


Figure 2.2. Preliminary results of four contaminants in avocado puree. Each method was submitted to 30 extraction cycles to evaluate the reproducibility of the DI-SPME protocol using the FWM.

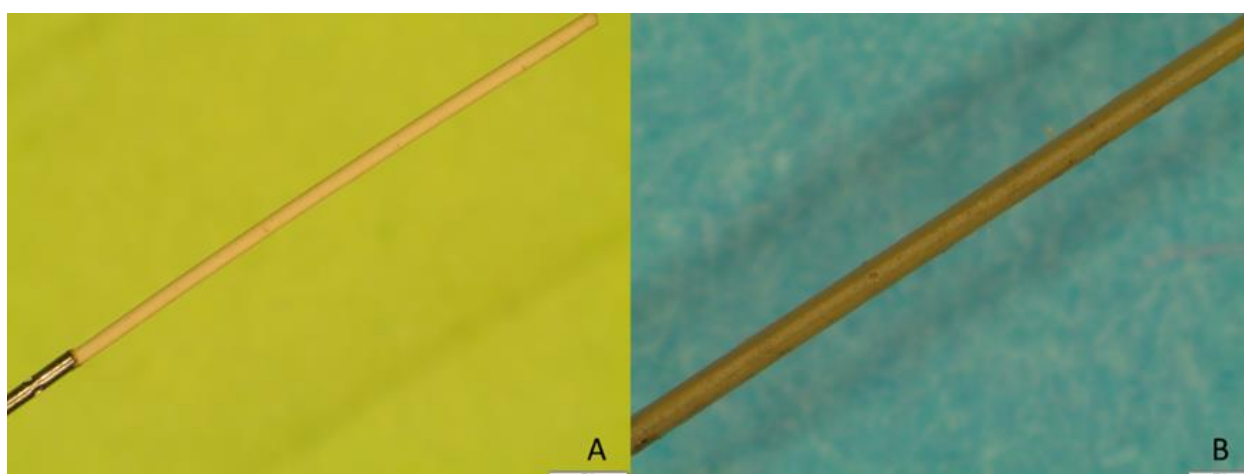


Figure 2.3. Microscopic pictures of a PDMS/DVB overcoated fiber before and after extraction in avocado using the CTC PAL3 Autosampler System with FWM. **A.** Overcoated PDMS fiber before avocado extraction (0.6X magnification). **B.** Overcoated PDMS fiber after 30 avocado extractions using FWM method *Rinse* (fiber tip, 2.5X magnification).

After a closer inspection of the FWM, it was obvious that this module was primarily designed for the cleaning of liquid syringes. The FWM has a large hole in the liner to allow the solvent from the pumps to go to the waste line; even though one of the designed methods entails filling the liner with solvent and immersing the fiber, the hole is located in the middle of the liner, which means that only half of it will be full, and there are no going to be a completed dipping of the fiber, even with maximum penetration. Since the fiber can never be fully immersed in the cleaning solvent, it does not benefit from the maximum flow in the liner when the pump is on (Figure 2.4).

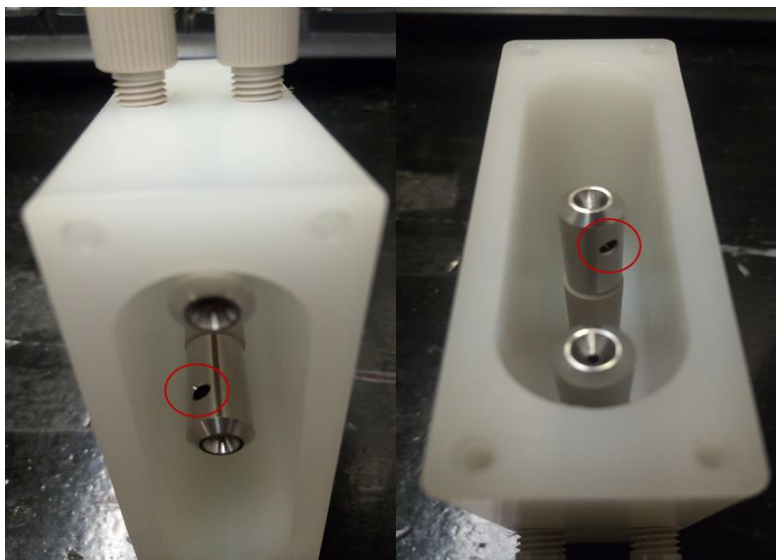


Figure 2.4. FWM photographs showing the holes within the liners. This design presumably allows the solvent that comes from the pump to move to the waste line without coming out from the top of the liner.

2.3.2. Different strategies for the cleaning of the fiber in the avocado extraction

Although the FWM did not work as expected in removing the matrix from the surface coating, the CTC PAL3 autosampler system offered other modules that improved the present methodology for

extracting targeted contaminants from avocado. Ultimately, the developed method or protocol must be robust enough to enable high throughput during the whole process, and the autosampler featured two new modules that were beneficial towards this end: a vortexer, and an SPME fiber conditioning chamber. These features expanded the range of the automated method's available functions, which proved to be advantageous because it directly contributed to the development and validation of a quantitative method for this complex matrix.

One of the disadvantages of the method developed by De Grazia *et al.* is that it is a proof of concept, which means that the samples must be mixed prior to extraction and outside of the automated procedure in a multipurpose vortexer due to the fact that the autosampler used in their method is only equipped with a six-vial agitator/incubation module. Another advantage of this new CTC PAL3 system is that the vial trays now allow up to 45 positions for vials at the same time in one tray (if using 10 mL vials), which makes it possible to conduct more than 10 extractions per day. As such, tests were conducted to determine whether this new module could be used to run all the samples at once, and not just 10 at a time.

For these experiments, 100 avocado puree samples with the same concentration (4.8 $\mu\text{g/g}$) were prepared on the day of the test. Additionally, the fiber was visually evaluated every 10 runs while the instrument was performing QCs.



Figure 2.5. Microscopic pictures of a PDMS/DVB overcoated fiber used in avocado extraction. **A.** PDMS overcoated fiber prior to avocado extraction (3.2X magnification). **B.** PDMS overcoated

fiber after 30 cycles of avocado extraction (2.5X magnification). C. PDMS overcoated fiber after 90 cycles of avocado extraction (3.2X magnification).

Five pesticides were used in these tests (

Table 2.1), along with the same DI-SPME methodology, cleaning solutions, times, and agitation speeds used by De Grazia *et al.* While initial visual inspections of the fiber showed that the methodology was working (Figure 2.5B), when the extraction cycles were analyzed it became clear that preparing all the samples at once was not suitable for this type of analysis (Figure 2.5C).⁴²⁻⁴⁴ The RSD values for the avocado analysis of these 5 pesticides were above 50% (Figure 2.6), while the RSD values for the instrumental QCs in water were all below 20% for the 100 extractions. Furthermore, although the autosampler used in De Grazia *et al.*'s proof of concept was obtained from CTC Analytics, it was necessary to assess the agitation speeds and the temperatures used in the development of the CTC PAL3 methodology.

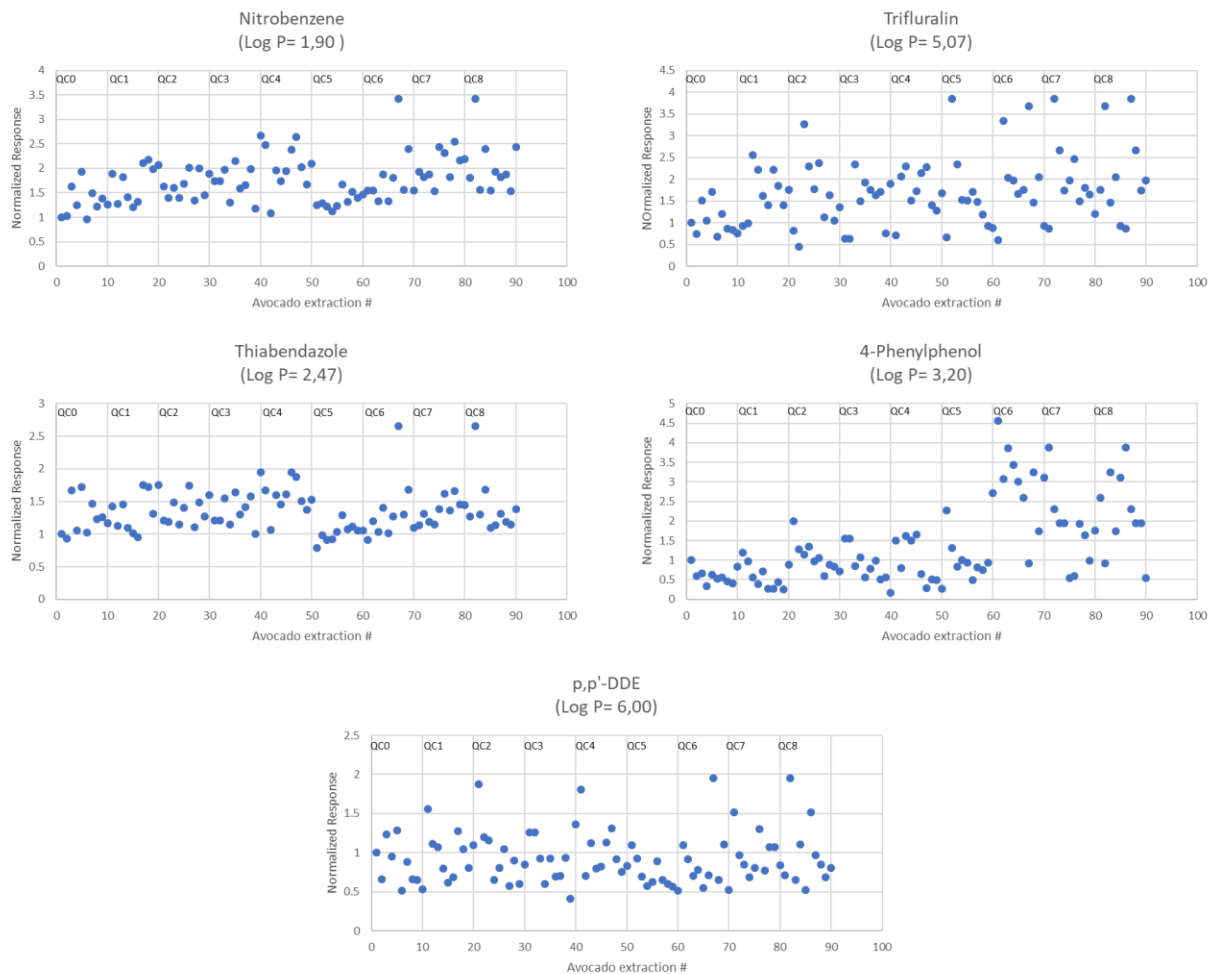


Figure 2.6. Contaminant extraction reproducibility in avocado after 90 extraction cycles.

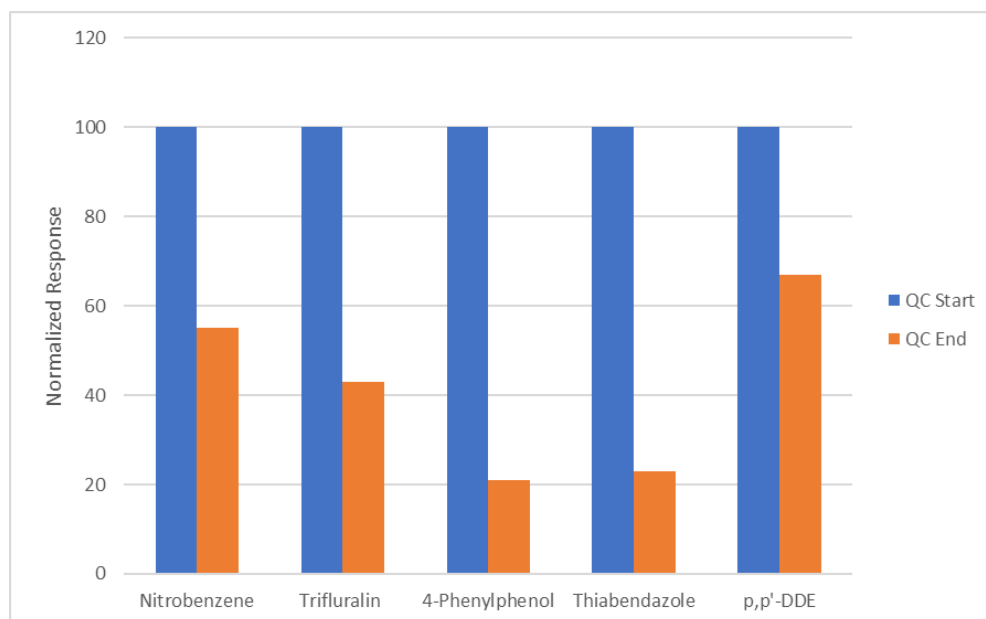


Figure 2.7. Extractions using a PDMS/DVB overcoated fiber performed in QC solutions comprised of pesticides spiked in ultra-pure water (3 ppm) in order to evaluate fiber performance before and after avocado extraction.

Consequently, the decision was made to evaluate the agitation speeds of the extraction and cleaning processes (this include rinsing and washing pre- and post-desorption). After meaningful discussion, it was decided that, even though De Grazia *et al.*'s proof of concept work used a CTC Analytics system, it was necessary to examine the conditions used for the extraction and cleaning steps in the proposed DI-SPME protocol. To this end, five different agitation speeds were examined for the extraction/cleaning process, taking into account that low speeds in the extraction process usually yield less matrix attachment to the surface of the SPME device when working with fatty matrices.^{5,44} In contrast, the use of higher speeds for the cleaning process can be the key to good fiber cleaning prior to thermal desorption.⁵

Five speeds at 25 rpm increments between 350 and 450 rpm were tested for avocado puree extraction. Similarly, the cleaning speed -including both the pre-desorption rinsing step and the post-desorption washing step- was assessed by examining a range of 475 to 600 rpm in 25 rpm increments, with the extraction speed being held constant at 425 rpm as detailed in the original protocol. These speeds were maintained during the incubation period, as well as for the extractions

from the avocado puree. For every speed tested, the used fiber and the liner of the instrument were visually evaluated in order to gain a better understanding of the cleaning process.

For the selection of the agitation speeds that were going to be use in an experimental design, the values of the relative standard deviations (RSDs) were use as the measurement of the precision of the methodology employed. These values were obtained out of ten consecutives extractions of pesticides from avocado samples, testing each speed one day at a time and the speeds selected were 375, 400, and 425 rpm, as the RSDs values showed are mostly below 25% for all of the five analytes. The completed results are shown in Table 2.3.

Table 2.3. RSD values in the optimization of the agitation speed in avocado extraction. Cleaning speed was held constant at 425 rpm.

Agitation Speed (rpm)	RSD (<i>n</i> = 10)				
	Nitrobenzene	Trifluralin	4-Phenylphenol	Thiabendazole	p,p'-DDE
350	22.2	31.7	31.5	21.1	31.3
375	8.5	39.8	20.5	10.8	20.7
400	15.8	37.7	14.4	17.5	16.4
425	20.4	18.9	15.5	10.7	13.4
450	25.3	20.2	34.7	18.4	28.3

* The highlighted speed values were selected based on RSD values below 25% for all or most of the five analytes.

There was variability in the extraction of the compounds, with the RSDs for four of the five compounds dropping below 20%, namely nitrobenzene, 4-phenylphenol, thiabendazole and p,p'-DDE; despite trifluralin RSD values for 400 and 425 rpm, these speeds were selected as a factor to optimized in a Central Composite Design (CCD) experiment.

Agitation speeds ranging from 475 to 600 rpm were examined for the cleaning process (rinsing and washing, pre- and post-desorption), with speeds increasing in increments of 25 rpm each day. After each test, the fiber was optically inspected, as this is an important tool in understanding how

the cleaning affects the fiber. For these tests, the extraction speed was left at 425 rpm, which is the same speed used in the protocol described by De Grazia *et al.*

The best RSDs obtained for the extraction of pesticides from avocado samples were 475, 500, and 525 rpm with the agitator/incubator on for 5 seconds and off for 1 second. The completed results are shown in Table 2.4.

Table 2.4. RSD values in the optimization of the agitation speed in avocado extraction.

Extraction speed was held constant at 425 rpm.

Agitation Speed (rpm)	RSD (n = 10)				
	Nitrobenzene	Trifluralin	4-Phenylphenol	Thiabendazole	p,p'-DDE
475	15.8	27.7	14.4	17.5	16.4
500	9.5	19.8	11.5	17.8	20.7
525	15.8	17.1	24.4	17.5	16.4
550	20.4	18.9	25.7	19.7	33.9
575	22.2	33.3	31.9	40.9	21.3
600	32.2	41.7	51.5	28.1	43.3

* The highlighted speed values were selected based on RSD values below 25% for all or most of the five analytes.

Again, there was variability in the extraction of the compounds, with the RSDs for three of the five compounds falling below 20%, namely nitrobenzene, thiabendazole and p;p'-DDE. As with the previous tests, despite the fact that for some speeds the RDS values obtained for trifluralin and 4-phenylphenol were above 20%, these speed values as a factor to optimized in a Central Composite Design (CCD) experiment.

After these preliminary trials the three different speeds capable of yielding RSD values below 20% for almost all of the pesticides in both the extraction/incubation process (375,400, and 425 rpm) and the cleaning process, which consists of the rinsing and washing steps (475,500, and 525 rpm) were evaluated using a simple experimental design, which provided an insight into the best

conditions for the whole process of performing extractions from avocado samples using the CTC PAL3 system.

The Central Composite Designs (CCD) consisted of the following:

Two Factors: Factor A: Extraction speed. Factor B: Cleaning Speed (rinsing and washing steps as one).

The values use in this experimental design were:

Factor A. Extraction Speed				
-1.67	-1	0	+1	+1.67
358	375	400	425	442

Factor B. Cleaning Speed				
-1.67	-1	0	+1	+1.67
458	475	500	525	542

The randomized experiments were performed following the run log showed in Table 2.5.

Table 2.5. Experimental design randomized run log for the extraction of 5 pesticides from avocado puree.

Run Order	Extraction Speed	Cleaning Speed
16	0	-1.67
13	0	0
18	1	-1
12	-1.67	0
14	1	1
6	0	0
4	0	+1.67

5	1	1
17	0	0
2	-1	-1
3	-1	-1
15	0	0
9	0	0
8	0	0
11	1	-1
10	-1	1
1	+1.67	0
7	-1	1

After the CCD was performed, the response surface was used to analyze the data (Figure 2.8). The data analysis revealed that the higher speeds provided the best results for both the extraction and the cleaning steps. Therefore, the selected values for the avocado extraction were:

Extraction speed: 425 rpm using the agitator module in the CTC PAL3 system.

Cleaning speed: 525 rpm using the agitator module in the CTC PAL3 system.

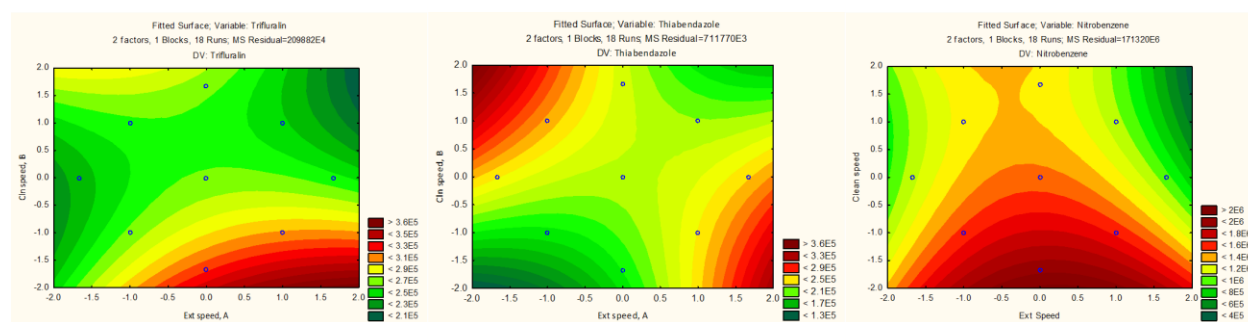


Figure 2.8. Response surface area plots for 3 of the 5 pesticides used in the avocado extraction. The pesticides shown range from a high to a low Log P.

Subsequently, a new round of analyses were performed using these new agitation speeds for the extraction and the cleaning processes. This time, 10 samples were prepared each day, with QCs being performed at the beginning and end of each 10-extraction cycle for 11 cycles (110 extractions in total). The results of these tests showed RSDs of less than 20% for all of the pesticides in the avocado samples (Figure 2.9), although the visual inspection of the fiber revealed that some surface fouling was still occurring ((Figure 2.11). Nonetheless, all 5 compounds analyzed in these tests had RSDs of less than 20% (Figure 2.9), and the instrumental QCs showed good instrumental reproducibility with RSDs below 10% for all the pesticides (Figure 2.11).

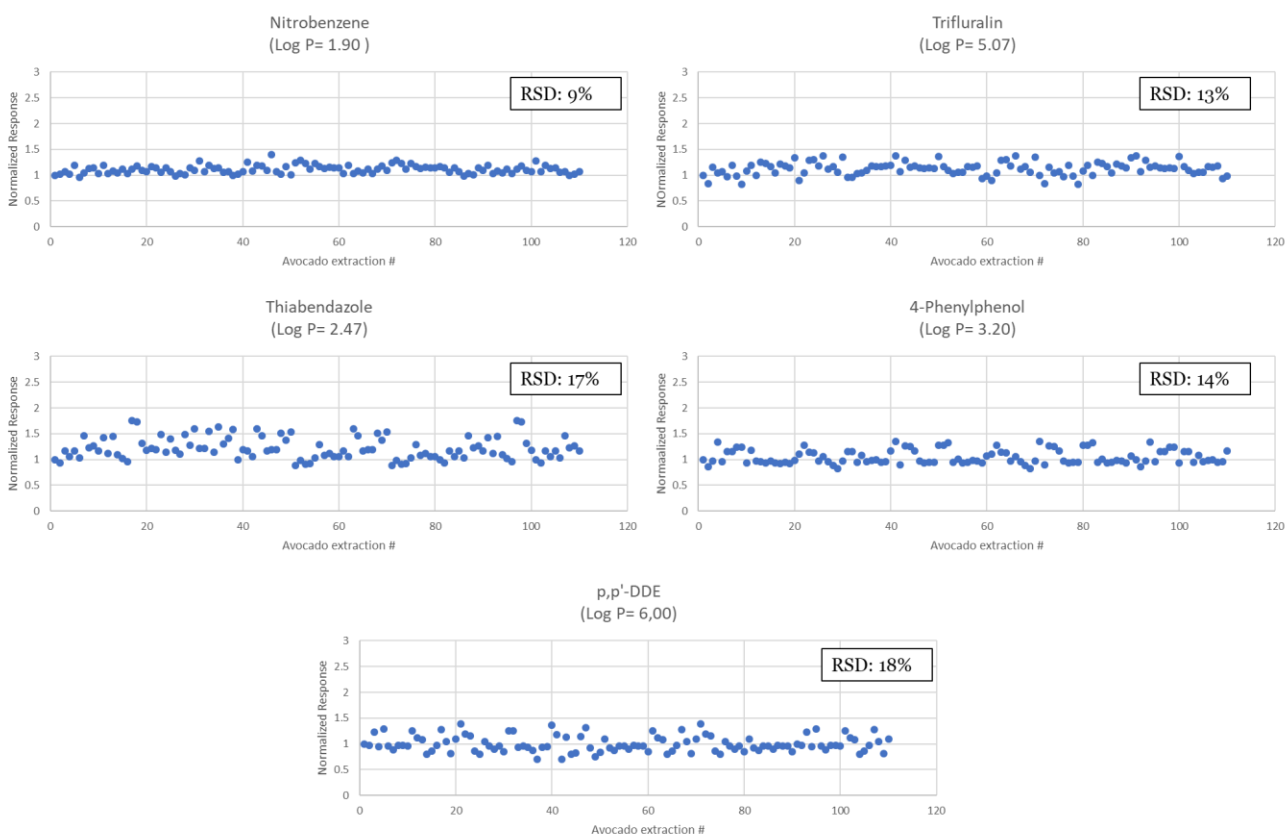


Figure 2.9. Contaminant extraction reproducibility in avocado puree after 110 extractions with new speeds for the incubation/extraction step and cleaning process (rinsing and washing).

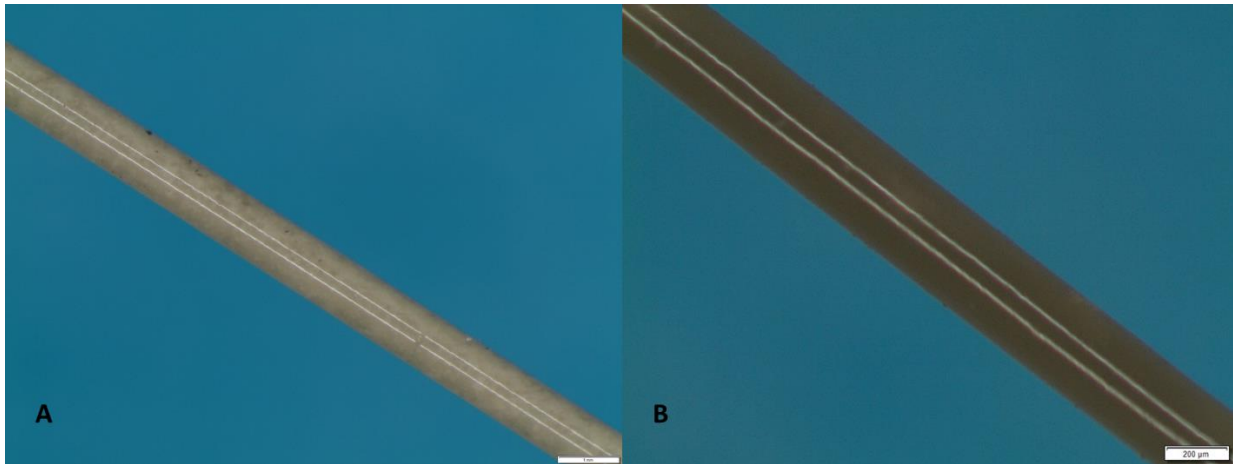


Figure 2.10. Microscopic pictures of a PDMS/DVB overcoated fiber used in over 110 consecutive extractions from avocado puree. **A.** PDMS/DVB overcoated fiber after thermal conditioning and before extraction from avocado puree (2.5X magnification). **B.** PDMS/DVB overcoated fiber after 110 consecutive extractions from avocado puree (3.2X magnification).

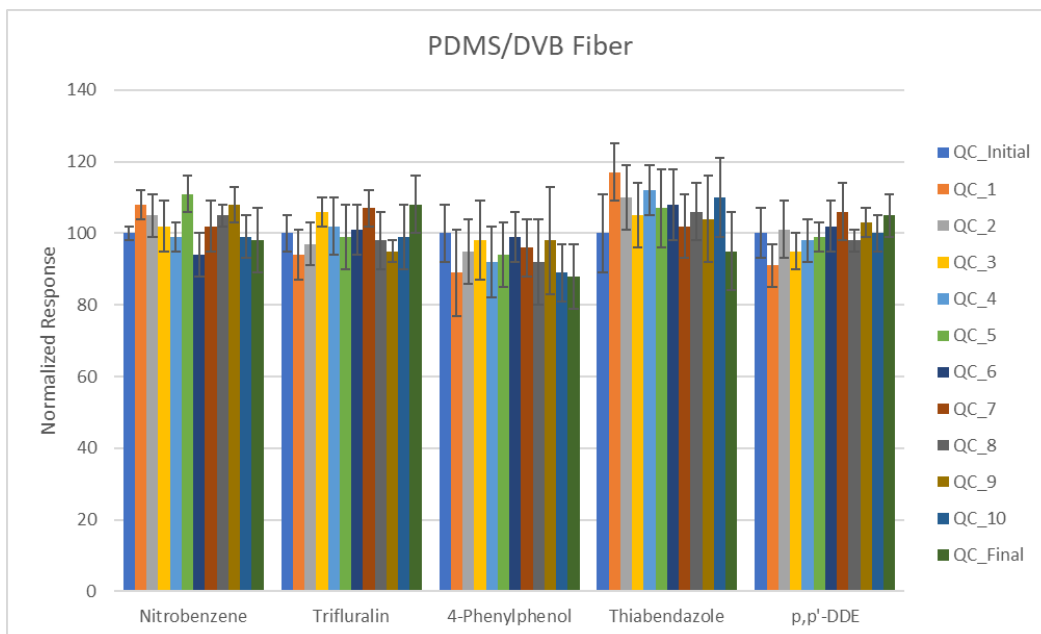


Figure 2.11. Instrumental QCs for the consecutive extractions from avocado puree. The QCs were performed after every 10 extractions using a PDMS/DVB fiber and a water sample spiked with a concentration of 3 ppm of the pesticide mixture.

2.4. Conclusions

The introduction of a Fast Wash Module to the automated DI-SPME protocol for analyses of fatty matrices proved to be unhelpful with respect to the cleaning process, possibly due to the fact that it was primarily designed to facilitate the fast cleaning of liquid syringes. However, the introduction of the FWM is a step in the right direction, as the development and evaluation of new technologies that can enhance the use of SPME for the analysis of complex matrices is critical, not only in the food analysis field, but also in the biological and environmental fields.

In addition, this chapter detailed a new evaluation of the optimal agitation conditions for the proposed protocol, which confirmed that the DI-SPME protocol is suitable for use in automated analyses of fatty matrices provided the lifespan of the device (i.e., fiber, blade, thin film) can be preserved. In this regard, the use of an organic solvent as a cleaning solution for the SPME device was confirmed as one of the most important parameters in the re-evaluated SPME protocol. The use of ultra-pure water as a solvent for fatty matrices caused irreversible damage to the lifespan of the fiber within five extractions, which was the result of the additional thermal desorption step. Thus, acetone was identified as the best solvent for this application, as it helped to remove the oily layer formed on the fiber while also preventing any significant analyte loss; however, it is important to highly that the use of organic solvents in SPME extractions are restricted, due to the fact that an organic solvent can act as a new extractive phase in the extraction procedure and can decrease considerably the amount of analyte extracted by the fiber, compromising sensitivity of the methodology.

Even though the inter and intra-reproducibility was not directly evaluated, the RSDs values from the five analytes were below 20% after more than ten days of consecutive extractions from avocado samples prepared daily for every ten extraction cycles using the same SPME fiber showing the reproducibility achieved with the presented DI-SPME methodology.

Ultimately, the DI-SPME protocol re-evaluated in this chapter offers a powerful, green analytical approach that can be used for the targeted and untargeted analysis of contaminants in fatty matrices.

Chapter 3. Analysis of different types of contaminants in avocado using Solid-Phase Microextraction (SPME) coupled with gas chromatography mass spectrometry (GC-MS)

3.1. Introduction

The analysis of pesticide and contaminant residues in foodstuffs is a challenging field that involves the simultaneous identification and quantitation of trace amounts of a wide range of hazardous chemicals. Indeed, over 1000 classes of pesticides with different physicochemical properties are applied to agricultural crops each year in order to control undesirable pests. As such, the determination of these trace amounts and the development of methods that are robust, accurate, and green is one of the most interesting challenges in contemporary analytical chemistry.^{5,39,45} However, the development of multi-residue methods that allow for the proper identification and quantitation of a large number of pesticides within a single analysis is difficult due to the complexity of the samples under study and the large amount of chemicals or analytes that can be present in these heterogeneous matrices.

Usually, the very first step in developing a multi-residue method is to select a sample preparation methodology, which will largely depend on both the analytes and sample matrix under study. Consequently, it is useful to strategically develop sample preparation methods that take into consideration the fat content in the matrix. ‘Fatty’ matrices are generally classified as those with a fat content of greater than ~5%^{43,45}, making them the most complex and challenging samples for analysis due to the large range of matrices that fall into this category.³⁹ In particular, the extraction of contaminants from fatty matrices is often susceptible to the coextraction of lipids, which may constitute a serious source of instrumental contamination, thus compromising the methodology accuracy and precision.⁴⁵⁻⁴⁷

To date, researchers have developed and implemented various strategies for avoiding the co-extraction of lipids in multi-residue analyses of pesticides in fatty matrices, including liquid-liquid

extraction (LLE), gel-permeation chromatography (GPC), matrix solid-phased extraction (MSPE), supercritical fluid extraction (SFE), and QuEChERS.^{45,47-51}

In the previous chapter, direct-immersion solid-phase microextraction (DI-SPME) was successfully verified as a valid technique for the analysis of contaminants in fatty matrices, such as avocado (fat content up to 30%). Specifically, the results of these tests confirmed that the combined use of a PDMS overcoated fiber and a well-tested cleaning methodology can allow a single fiber to be used for up to 100 extraction cycles. In addition, the results demonstrated that the developed method allows for a range of analytes to be extracted with minimal sample preparation and a fully automated SPME protocol.

The present chapter builds on the work presented in the prior chapter by focusing on the development of a fully automated DI-SPME protocol for quantitating contaminants in avocado samples using CTC Analytics' CTC PAL 3 System. The method was validated via SPME matrix-match calibration curves, as well as by determining the Limits of Detection (LODs), Limits of Quantitation (LOQs), as well as linearity. Overall, the automated DI-SPME protocol proved to be suitable for use with fatty matrices, and the conjoining of an automated system like the CTC PAL3 system with SPME as a sampling preparation technique further enabled high-throughput analysis, while also reducing the time-consuming sample preparation steps required by other analytical techniques for fatty matrices.

3.2. Experimental

3.2.1 Chemicals and materials

All chemicals—namely, nitrobenzene, 2,6-dinitrotoluene, trifluralin, 4-phenylphenol, diazinon, parathion, parathion methyl, p,p'-dichlorodiphenyldichloroethylene (p,p'-DDE), thiabendazole, and 2-phenylphenol—were supplied by Sigma Aldrich (St. Louis, MO, USA), while HPLC-grade methanol, acetonitrile, and acetone were purchased from Fisher Scientific (Bartlesville, OK, USA). Purified water was obtained using Milli-Q systems (Waterloo, ON, Canada). A stock

solution with concentrations ranging from 5 to 800 ppm for each chemical was prepared in methanol and stored in the refrigerator at $-30\text{ }^{\circ}\text{C}$ until use. Different dilutions of this solution were prepared as needed based on the concentration levels required for the matrix used (avocado). PDMS/DVB overcoated fibers (65 μm coating thickness + 10 μm PDMS layer) and PDMS/DVB fibers (65 μm coating thickness) were purchased from Supelco (Bellefonte, PA, USA). Samples of organic avocado fruits (*Persea americana* ‘Hass’) were purchased in local grocery stores and were cut into pieces and homogenized with an electric blender prior to SPME extraction.

3.2.2. SPME procedure

For the quality control (QC) analyses, an instrumental QC using a new gas-generating vial with McReynolds solutions was developed and implemented (Chapter 4), using a PDMS/DVDB fiber for this sole purpose.

For analyses in avocado samples, 20 g of homogenized avocado pulp was weighed in a 40 mL amber vial and combined with 400 μL of a solution of standards ranging from 5 to 800 mg/L. The samples were then left overnight (10-12 hours) under constant stirring (1500 rpm) by means of a MultiTube Vortexer (Fisher Scientific, Waltham, MA, USA). Next, 2.1 g of the spiked fruit pulp was transferred to a 10 mL amber vial and diluted with 4.9 mL of deionized water (dilution level 30-70%) to produce final matrix concentrations ranging from 0.03 to 4.8 $\mu\text{g/g}$. Initially, the SPME extraction procedure consisted of the following steps: 1) incubating the sample for 1 min at $35\text{ }^{\circ}\text{C}$; 2) directly exposing the fiber to the sample for 40 min at $35\text{ }^{\circ}\text{C}$ under agitation at 425 rpm; 3) 7 s of pre-desorption rinsing in acetone/water (9:1, v/v) at 525 rpm; 4) desorption in the injection port for 5 min at $270\text{ }^{\circ}\text{C}$ in splitless mode; and 5) 30 s of post-desorption washing in pure acetone at 525 rpm.

The avocado sample analyses were carried out in order to ensure that every randomized calibration curve showed a separation of the avocado samples by quality control (QC) analyses (in triplicate). A standard gas-generating vial containing McReynolds standards would be used for instrumental QCs along with the PDMS/DVB fiber. The responses obtained from the experiments were normalized according to the QC response in order to account for drifts in instrumental response.

3.2.3. GC/MS equipment and analysis conditions

GC/MS was performed using an Agilent 6890-5977A GC-MS (Mississauga, ON, Canada) that was equipped with a DB-5 MS column (30 m, 0.25 mm I.D., 0.25 μ m film thickness) (Agilent Technologies, Santa Clara, CA, USA). A SIM method was established using the m/z ratios and retention times (

Table 3.1) for the contaminants in the avocado samples, and instrumental QCs were conducted with the instrument in *full scan* mode. Helium (purity 99.999%) was used as a carrier gas, with a linear velocity of 1.5 mL/min. The oven temperature was set to 40 °C and held there for 2 min before being raised to 180 °C at 10 °C/min; finally, it was raised to 300 °C at 20 °C/min, where it was held for an additional 5 min. The injector was kept constant at 270 °C, the transfer line temperature was set to 250°C, the MS source was held at 230°C, and the MS quadrupole was set to 150°C. The instrument was controlled using a PC equipped with Agilent Masshunter Qualitative Analysis software. Lastly, these tests were conducted using a CTC PAL 3 Autosampler System equipped with a six-vial agitator/incubator, a vortexer, an SPME conditioning module, an SPME park station, and headspace and liquid-injection tools.

3.2.4. Statistical analysis

Microsoft Excel v16.14 was used for statistical analysis.

3.3. Results and Discussion

3.3.1. Evaluation of DI-SPME methodology for quantitative analysis in avocado puree.

In Chapter 2, the most appropriate conditions for the extraction of five analytes were established for the CTC PAL3 system's various modules.

Table 3.1 provides a list of the ten analytes used in this study. The introduction of an internal standard helped to correct and enhance the DI-SPME protocol's sensitivity and enabled proper figures of merit to be obtained in the quantitation of contaminants in avocado samples.

Table 3.1. List of analytes used for quantitation in avocado extraction using the CTC PAL 3 Autosampler System.

Pesticides	Molecular Weight (g/mol)	Log P (pH 7)	Retention Time (min)	m/z ratio	Boiling Point (°C)
Nitrobenzene	123.06	1.90	8.30	77	210.9
2,6-Dinitrotoluene	182.14	2.10	13.00	165	285.0
Diazinon	304.35	3.81	17.20	179	-
Parathion	291.26	3.83	21.30	291	150
Parathion Methyl	263.21	2.86	19.50	109	143
2-Phenylphenol*	170.20	2.94	13.80	170	283
Trifluralin	335.28	5.07	15.10	306	139.5
4-Phenylphenol	170.21	3.20	16.30	170	305.0-308.0
Thiabendazole	201.24	2,47	22.20	201	-
p,p'-DDE	318.02	6.00	23.40	246	336.0

* 2-Phenylphenol was used as an IS for all the contaminants. All physicochemical data was collected from the NIST Chemistry WebBook, online database (<https://webbook.nist.gov/chemistry/name-ser/>, Access on February 12, 2018).

As previously noted, the samples were prepared on the same day they were to be analyzed; as such, one matrix-match calibration was performed per day.

Since new contaminants were introduced during the development and validation of the methodology, it was also necessary to test the reproducibility of the analyses focusing on them. However, as the final objective of this study is to develop and validate a quantitative method, the reproducibility trials were done by spiking the avocado samples with relatively low concentrations

of these contaminants (final concentration samples: 1.5 $\mu\text{g/g}$). The reproducibility assessment entailed 25 replications of the extraction cycle, with 10 samples being prepared and analyzed per day (Figure 3.1 and Figure 3.2). RSDs of less than 20% were obtained for all of the compounds spiked in the avocado, with nitrobenzene and trifluralin returning RSDs of less than 10%. Unfortunately, the reproducibility evaluations were suspended at this point due to some minor instrumental failures. However, since the tests had demonstrated that the method provided good reproducibility for all compounds after 25 extraction cycles, the validation experiments were stopped at this point.

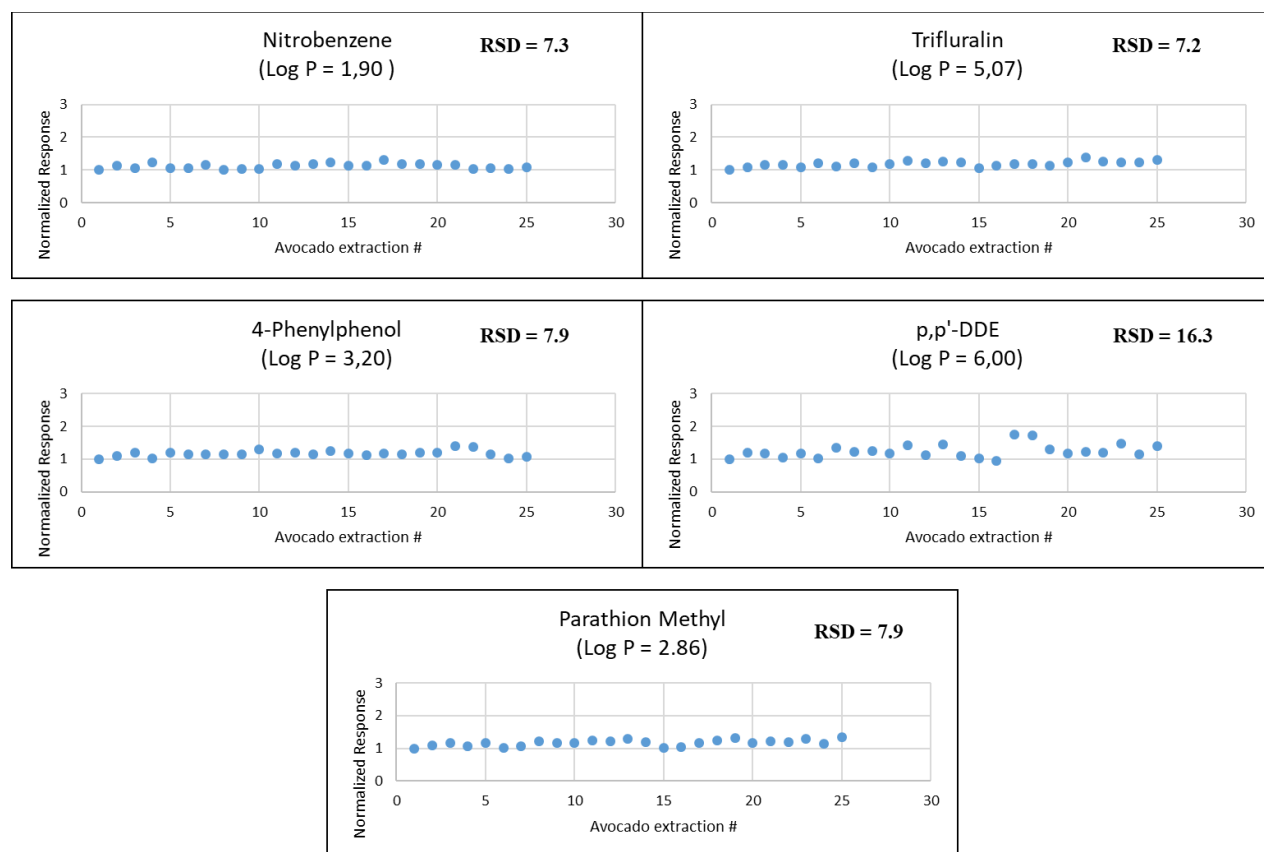


Figure 3.1. Series of 25 consecutive extractions from avocado puree using a PDMS overcoated fiber for five contaminants.

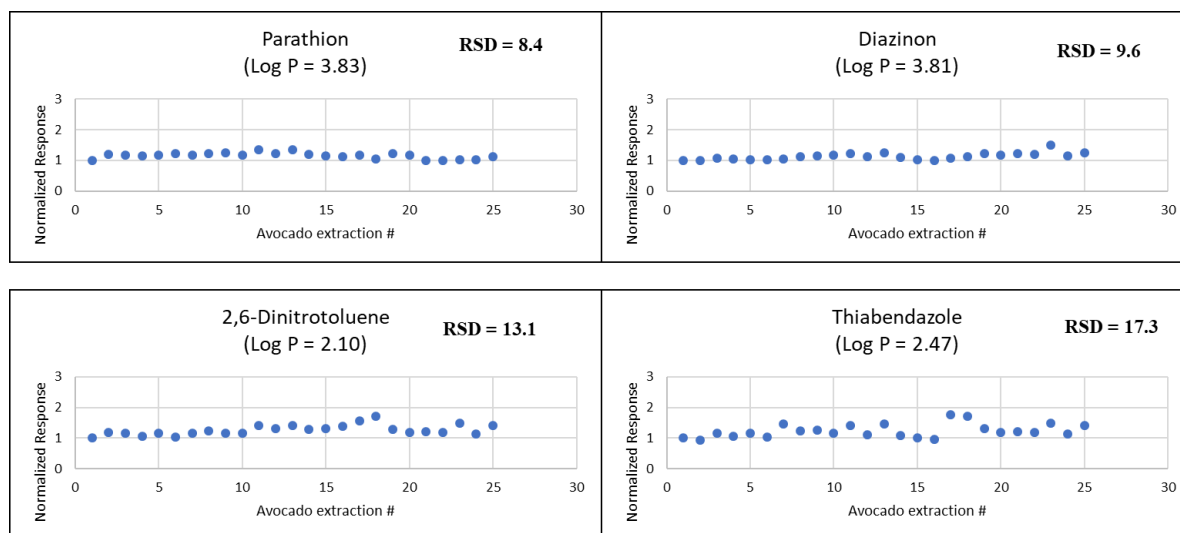


Figure 3.2. Series of 25 consecutive extractions from avocado puree using a PDMS overcoated fiber for four contaminants.

The fiber was also visually inspected as part of the cleaning-protocol optimization in order to evaluate the procedure's effectiveness. These inspections revealed that, even when the fiber maintained its performance during the extractions, its surface appeared to be affected by some degree matrix attachment to one particular part of the surface (Figure 3.3). Although visual inspection played a critical role in optimizing the extraction and cleaning protocol (Chapter 2), this localized accumulation of oily components from the matrix was not observed. As a result, the decision was made to perform visual inspections of the fiber after every extraction for ten cycles. This approach revealed that the localized accumulation of oily components tended to occur after the 10th extraction cycle; although this result can be considered negligible because it does not affect the reproducibility of the extraction, it was nevertheless taken into account before validating the quantitative method. After examining the steps used in the automated methodology more closely, the decision was made to slightly alter the amount of time the fiber was left in the agitator during the pre-desorption rinsing step. Initially, the agitator was programmed to be *on* for five seconds and *off* for two seconds during the incubation, extraction, rinsing, and washing steps. However, it was noticed that the agitator would not change its orbital shaking direction during this *on/off* cycle; since the rinsing step only lasted for five seconds, the agitator only used one direction during cleaning. To remedy this, the rinsing time was increased to seven seconds, with the agitator module being programmed to be *on* for three seconds and *off* for one second, thus guaranteeing a change of

orbital direction in the agitator module during the rinsing step. After applying these changes to the autosampler, the visual inspection process was again repeated, and DI-SPME protocol's reproducibility was re-assessed. The results showed a negligible change in the RSDs of the nine contaminants and a notable decrease of the localized accumulation pattern on the surface of the fiber (Figure 3.4).

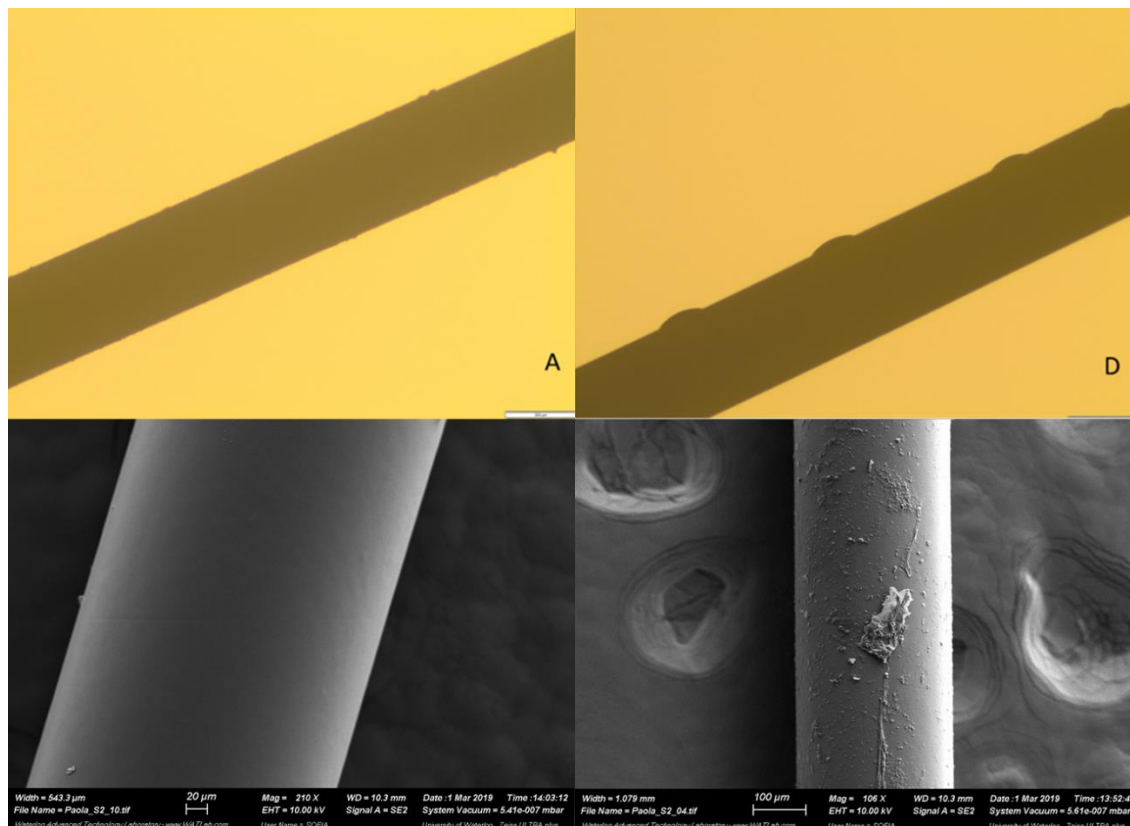


Figure 3.3. Microscopic images of a PDMS overcoated fiber. **A.** Contrast light image of a the PDMS overcoated fiber prior to extraction from avocado puree (2.5X magnification). **B.** SEM image of the PDMS overcoated fiber surface prior to extraction from avocado puree. **C.** SEM image of the PDMS overcoated fiber surface after 25 extraction cycles from avocado puree. **D.** Contrast light image of the PDMS overcoated fiber after 25 extraction cycles from avocado puree (2.5X magnification).

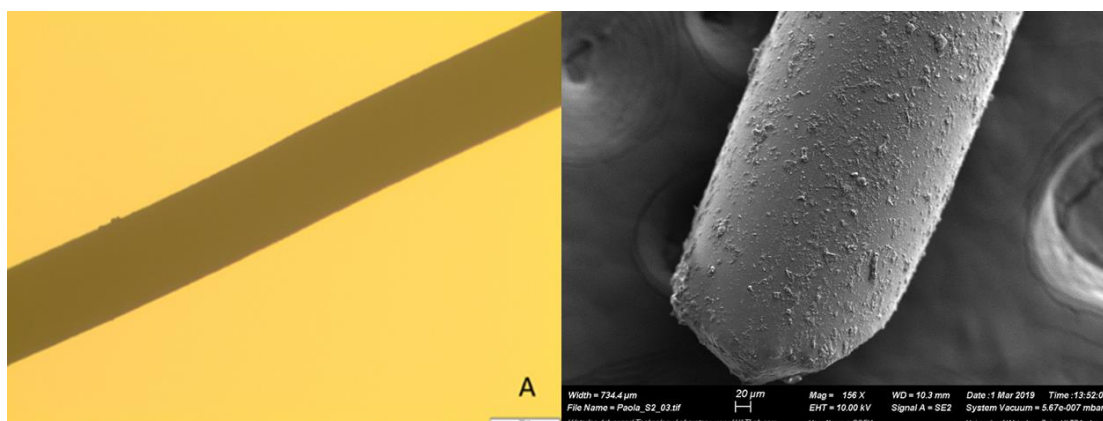


Figure 3.4. Microscopic images of a PDMS overcoated fiber. **A.** Contrast light image of the PDMS overcoated fiber after 10 extraction cycles from avocado puree using seven seconds as the rinsing time in the cleaning step (2.5X magnification). **B.** SEM image of the PDMS overcoated fiber surface after ten extraction cycles from avocado puree using seven seconds as the rinsing time in the cleaning step.

The initial trials for the matrix calibration curves, which were conducted using avocado samples spiked with concentrations ranging from 0.03 $\mu\text{g/g}$ to 4.8 $\mu\text{g/g}$, showed limits of detection of above 0.05 $\mu\text{g/g}$ for some of the analytes in the samples. Since one of this study's main objectives was to demonstrate the advantages of using the DI-SPME protocol for quantitative analyses of avocado samples, a small volume of organic solvent was added to the dilution solution^{36,52} in order to evaluate whether this would help to release the contaminants bonded to the avocado matrix, thereby reaching the minimum concentration detected by the instrument. The avocado samples were prepared following the above-described method with a final concentration of 1.5 $\mu\text{g/g}$. The dilution solutions were prepared using methanol and acetone as the evaluation solutions, which were spiked into water at concentrations ranging from 1-10%. A comparison of the different dilution solvents used in this evaluation is presented in Figure 3.5. Although extraction efficiency and extraction volume increased for some of the compounds using the described DI-SPME protocol, the RSD values in most of the experiments wherein a dilution solvent was combined with an organic solvent other than water were well above 20%. At the same time, this evaluation also showed that extraction efficiency decreased between 40% and 60% for compounds with LODs below 0.05 $\mu\text{g/g}$ when an organic solvent was added to the dilution solution during the first matrix

calibration curve trials. As previously noted, the use of organic solvents in the SPME protocol may lead to competition between the two phases in the extraction step, with the organic solvent acting as an antagonist to the SPME coating for the partitioning of analytes in the matrix.¹³ This interaction may explain the above results. Consequently, the decision was made to use water as the dilution solvent, and to evaluate the DI-SPME protocol's compatibility with avocado samples, even if the methodology could not achieve the LODs for some of the studied compounds presented in different studies where the matrix was water⁵³.

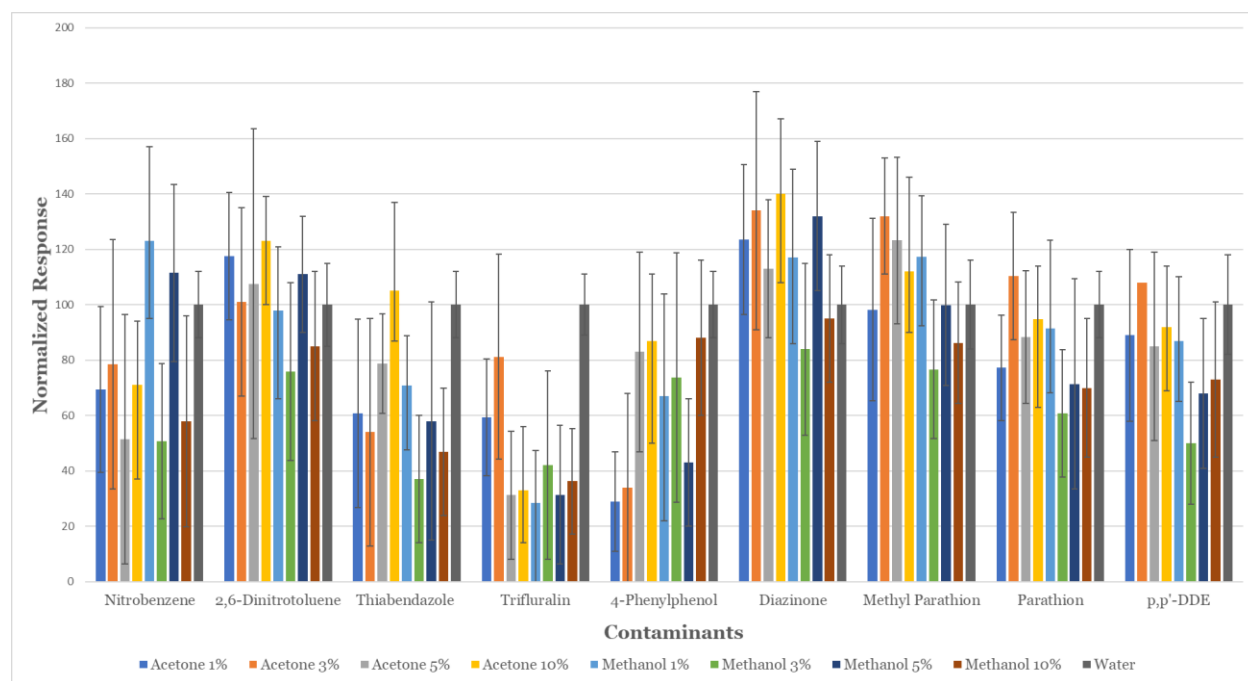


Figure 3.5. Comparison of extraction efficiency using different dilution solutions in avocado puree with a PDMS overcoated fiber ($n = 5$ samples).

As is shown in Figure 3.6 and Figure 3.7, the matrix-matched calibration curves displayed very good linearity ($R^2 > 0.9890$) for all nine of the analyzed compounds. In addition, very respectable figures of merit were obtained for the quantitation of the target analytes (Table 3.2), with the LOQs for all target analytes being determined at less than $0.05 \mu\text{g/g}$. The linearity of the calibration curves is slightly compromised when higher concentrations are used (final concentration of $4.8 \mu\text{g/g}$ in avocado) for almost all the compounds in the matrix; one the reasons behind the effect that

this concentration can be having over the linearity could be relate to an coextraction of the compounds from the fiber coating to the organic solvent use in the cleaning step (acetone), that was not accounted before due to the fact that the concentration use for the optimization of the cleaning of the fiber (Chapter 2) was conducted at this concentration in order to secure the extraction of all the compounds in the avocado matrix without bigger concerns about losing the analytes to the mentioned organic solvent.

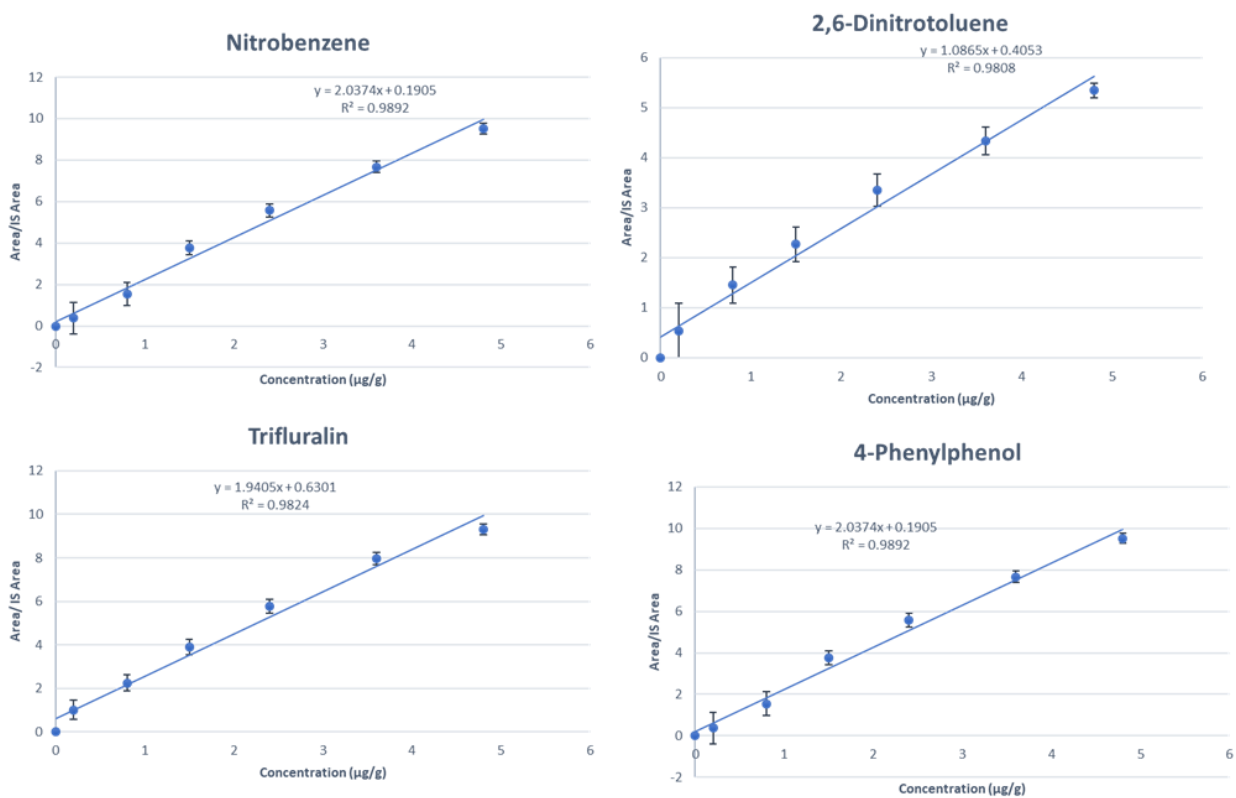


Figure 3.6. Matrix-match calibration curves of pesticides of 4 contaminants for quantitative analysis in avocado puree.

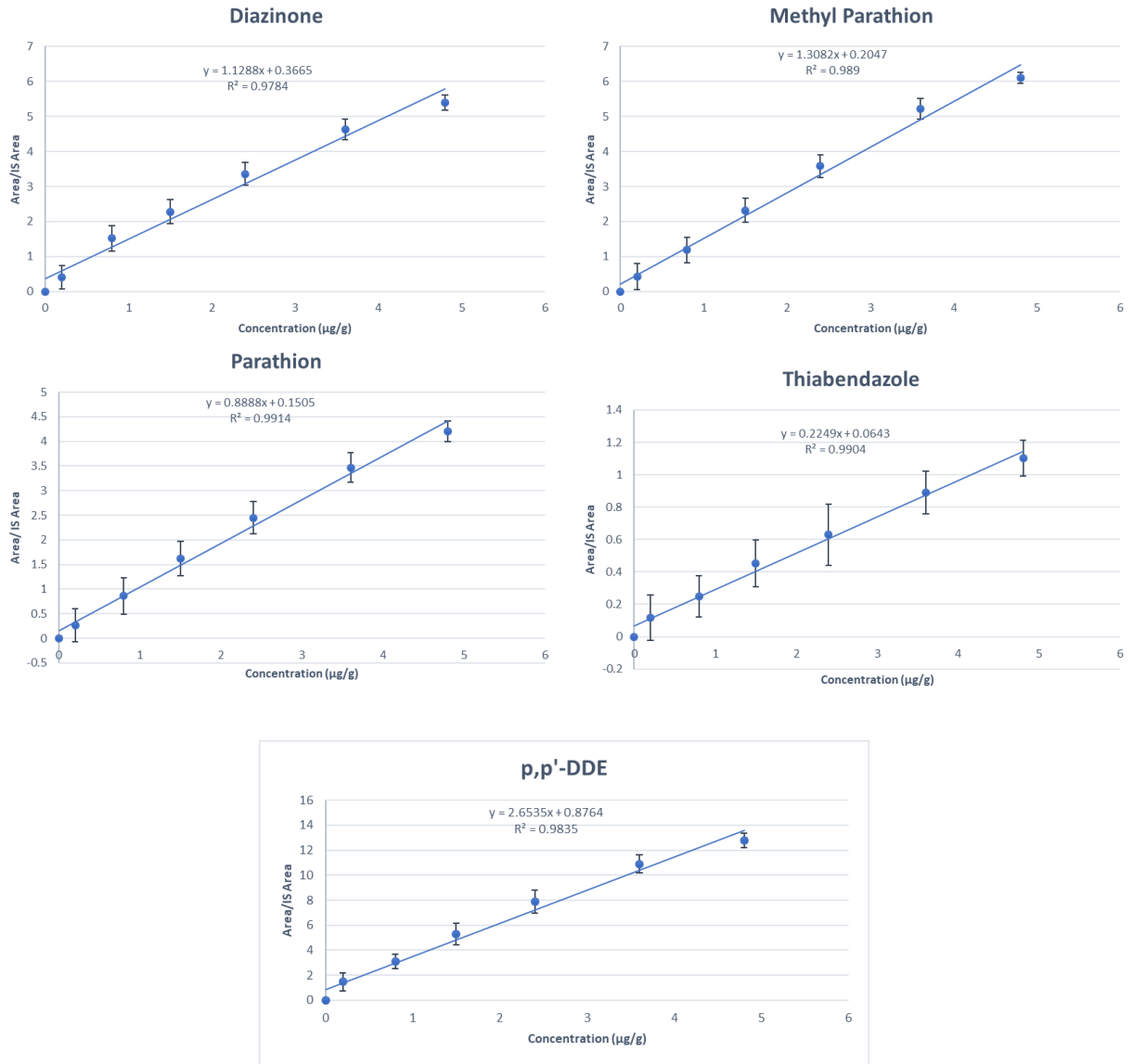


Figure 3.7. Matrix-match calibration curves of 5 pesticide contaminants for quantitative analysis in avocado puree.

Table 3.2. Figures of merit for validation of the DI-SPME protocol in avocado pure using GC/MS.

Pesticides	Slope	Intercept	R²	LODs (µg/g)	LOQs (µg/g)	Accuracy (n=3), %	Precision (n=3), %
Nitrobenzene	2.0374	0.1905	0.9892	0.05	0.08	94.5	8.5
2,6-Dinitrotoluene	1.0865	0.4053	0.9808	0.05	0.08	87.9	15.4
Diazinone	1.1288	0.3665	0.9784	0.03	0.05	102.2	14.3
Parathion	0.8888	0.1505	0.9914	0.03	0.05	109.1	16.8
Parathion Methyl	1.3082	0.2047	0.9890	0.03	0.05	98.9	12.6
Trifluralin	1.9405	0.6301	0.9824	0.05	0.08	89.5	9.7
4-Phenylphenol	2.0374	0.1905	0.9892	0.08	0.1	83.7	18.6
Thiabendazole	0.2249	0.0643	0.9904	0.08	0.1	85.8	17.4
p,p'-DDE	2.6535	0.8764	0.9835	0.03	0.05	105.9	10.1

Ultimately, the RSD values achieved for all the concentrations of the analytes in the matrix-matched calibration curves were below 20% and the values of accuracy and precision of the method were between 80-120% and 2-20%, respectively.

Despite the fact that these compounds can be quantitated in avocado puree, these LOQs cannot be properly compared with the maximum residue limits (MRLs) in the real data because, at this point, the avocado is considered one of the cleanest food commodities, with MRLs levels ranging from 0.5 to 75 mg/g for a few contaminants and metals, none of which includes the analytes studied in this chapter.⁵⁴ However, it is important to mention that the LODs and LOQs values found in this DI-SPME methodology are far below the ones stated for some analytes considered contaminants in the avocado matrix. As noted earlier, real avocado samples were purchased in stores in the Waterloo area and analyzed using the above-described protocol; and yet, none of the evaluated samples presented any of the compounds selected for this study, thus confirming the avocado's reputation as a relatively 'clean' food.

3.4 Conclusions

In this chapter, the optimized DI-SPME protocol was successfully implemented for the extensive quantitative analysis of nine targeted contaminants in avocado puree. The CTC PAL3 Autosampler System proved to be a convenient and powerful tool in the development and validation of a targeted quantitative method with decent figures of merit for analyses of high-fat matrices. It is important to point out that, even though the RSDs for some compounds were above 10%, the difficulties generated due to the nature of this matrix makes the developed method a suitable candidate for the quantitative analysis of different fatty matrices.

Furthermore, the results also confirmed the advantages related to the application of a PDMS layer on the solid porous coating (PDMS/DVB). This advance in SPME-coating technology has once again been proven as a potentially powerful tool for future applications dealing with complex matrices, as the inertness and smoothness of the PDMS layer allows for “easy” cleaning of the coating surface via a convenient automated protocol, which allows the fiber to be restored to an almost original condition.

Overall, the figures of merit obtained in this DI-SPME methodology for the analysis of multiple contaminants in avocado are respectable with good linearity ($R^2 > 0.98$), LOD and LOQ values that range between 0.03-0.08 $\mu\text{g/g}$ and 0.05-0.1 $\mu\text{g/g}$, respectively; and RSD values ranging between 5-20% for all the compounds analyzed. At the same time, the use of an internal standard for this type of multi-residue analysis in such complicated matrices can enhance the sensitivity achieved in the SPME methodology and help to decrease the uncertainty caused by the complexity of the matrix studied.

Chapter 4. New type of gas generating vial standard for SPME high-throughput analysis using thin film membrane.

4.1. Introduction

Solid-phase microextraction (SPME) is a sampling/sample preparation technique comprehensively described in the literature that has been widely accepted in different fields of analytical chemistry due to its easy handling, minimization of organic solvent consumption, and short sample preparation^{5,12}. At present, several calibration methods have been developed for both kinetic and equilibrium modes of SPME.^{12,55} Common calibration methods include pre-equilibrium extraction, equilibrium extraction, diffusion-controlled calibration, and kinetic calibration^{12,16,55}. The last method, henceforth named “in-fiber calibration,” is based on the simultaneous desorption of an internal standard previously loaded on the coating and extraction of the target analyte from the sample matrix.⁵⁶ The kinetic calibration approach is especially useful for *on-site* and *in vivo* investigations where there are difficulties adding the standard to the sample matrix or controlling environmental conditions.^{5,12,55}

The critical parameter that needs to be controlled is the amount of internal standard loaded onto the fiber coating. It should be at a level not as low as to cause detection problems, or as high as to overload the detector. It has been previously described that even for extremely short extraction times, large amounts of standard are loaded onto the fiber coating by headspace extraction of pure standards in a vial.^{5,12} A plausible solution was first proposed by Wang *et al.*,¹⁵ an approach that consists of spiking a few milligrams of standard into a predetermined amount of pump oil placed in a sealed vial. It has been proved that this experimental setup provides an excellent standard generator for over a 100 extraction/injection cycles using PDMS fibers, an essential feature when processing a large number of samples. Due to the low distribution coefficient that exists between headspace and the pump oil, a considerable decrease in headspace concentration of standards can be obtained. A few applications regarding the development and use of such gas generating vials have been developed by Grandy, *et al.*,⁵⁷⁻⁵⁹ in which, the aforementioned vial consisted in a composite of spiked vacuum oil loaded to a styrene-divinylbenzene resin (SDVB); subsequently, the action of the vacuum oil and the resin help to control the concentration of analytes present in

the headspace of the vial. In fact, the new gas generating vial performance could achieve over 200 headspace extractions with less than 10% of concentration depletion of the standards in the vial.⁵⁷ Thin Film Microextraction (TFME) is an extension of the SPME technology and it was developed to address the limiting uptake rate and capacity sometimes observed with fiber microextraction.^{12,60,61} Compare to a SPME fiber, both the volume of extraction phase and the surface-to-volume ratio are significantly larger. TFME presents two configurations, the brush, developed for liquid chromatographic applications and can be easily automated and/or the membrane, that usually exhibit a larger surface-to-volume ratio and it is mostly used in gas chromatographic applications.^{53,61,62}

In the present work, a new type of gas generating vial is built using as composite to control the amount of standards present in the headspace a thin film membrane that combines a carbon fiber mesh coated with a mixture of a slurry mixture that contains polydimethylsiloxane (PDMS) and Hydrophilic-Lipophilic Balance (HLB) particles. The new vial is evaluated via consequently automated extractions using the CTC PAL3 autosampler system and it managed to achieve over 300 extractions using only one SPME fiber with depletion levels below 2% for all the standards spiked.

4.2. Experimental

4.2.1 Chemicals and materials

All chemicals, namely benzene, 2-pentanone, octane, 1-pentanol, and pyridine, were supplied by Sigma Aldrich (St. Louis, MO, USA). HPLC grade solvents, namely methanol, acetonitrile, and acetone, were purchased from Fisher Scientific (Bartlesville, OK, USA). The divinylbenze/carboxen/polydimethylsiloxane fibers (DVB/CAR/PDMS), 50/30 μm were purchased from Supelco (Bellefonte, PA, USA).

4.2.2. SPME procedure

For the SPME procedure, two gas generating vials were used in the evaluation of the thin film membrane as an appropriate composite for the gas generating vial. In this regard, the first vial was one of the original studied gas generating vial containing silicon diffusion pump oil and polystyrene/divinylbenzene (PS/DVB) particles and it was used in this study as instrumental quality control (QC); meanwhile, the second vial, the thin film membrane vial was built using ~4.25 g of a PDMS/HLB^{63,64} membrane introduced to an amber vial and spiked with 1 µL of each standard (pure) and it was left conditioning and equilibrating at 35 °C for 24 hours. The SPME procedure followed was the same one described in Grandy, *et al.*,^{57,58} in order to achieve similar conditions and as a result the comparison will be more accurate. Initially, the gas generating vial must be left in the incubator one hour prior to SPME extraction, after this time is complete the automated method consisted in: one-minute incubation time at 35 °C, followed by one-minute extraction time at 35 °C without agitation and finally three minutes of thermal desorption at the injector port. It was established that QC measurements (triplicate extractions) were going to be conducted every 25 extraction cycles of the thin film vial following the exact same extraction conditions until the fiber or the gas generating vial were showing significant depletion levels (> 10% concentration depletion).

4.2.3. GC/MS equipment and analysis conditions

An Agilent 6890-5977A GC-MS (Mississauga, ON, Canada) was used. The instrument was equipped with a DB-5 MS column (30 m, 0.25 mm I.D., 0.25 µm film thickness) (Agilent Technologies, Santa Clara, CA, USA). A *full* scan mode (40-250 *m/z*) was used for the evaluation of the durability and reproducibility of the new gas generating vial, for quantification purposes the *m/z* ratios and retention times were used (Table 4.1). Carrier gas was helium (purity 99.999%), at a linear velocity of 1.0 mL/min. The oven temperature program was set at 40 °C and held for 1 min, then raised to 60 °C at 5 °C/min to, then to 80 °C at 6 °C/min and finally to 230 °C at 30 °C/min held for 1 min. The injector was kept at 270 °C. The transfer line temperature was 250°C, MS source 230°C, and MS quadrupole 150°C. The instrument was controlled by a PC running Agilent Masshunter Qualitative Analysis. The autosampler use was the CTC PAL 3

System, and consisted of the six-vials agitator/incubator, a SPME conditioning module and the park tool with a SPME, headspace and liquid injection tools.

4.2.4. Statistical analysis

Microsoft Excel v16.14 were used for statistical analysis.

4.3. Results and Discussion

4.3.1. Evaluation of the vial reusability

The development of a calibration solution that can be reused several times is critical, especially for high-throughput applications such as determination of food authenticity or SPME fiber aging evaluation, in which over 300 analyses must be carried out.⁵ Consequently, in order to determine standard gas generator reusability, 325 cycles of 1 min headspace extraction were performed using a 50/30 μm DVB/Car/PDMS fiber. Automated SPME incubation/ extraction/ desorption/ fiber bake-out cycles, together with the programmed GC–MS analysis, did not exceed more than 15 min, with GC separation of analytes completed within 7 min. Additionally, in order to ensure that variations observed in the amount extracted were unrelated to fluctuations in the detector response and/or fiber deterioration, QC tests for both factors were run in parallel to one another at 25 injections (Figure 4.2).



Figure 4.1. Gas generating vial: silicon diffusion pump oil and PS/DVB particles (Left) and carbon fiber mesh coated with the PDMS/HLB (Right).

Table 4.1. McReynolds standard and physicochemical properties.

Analyte	MW (g/mol)	BP (°C)	Q mass (m/z)	t _R (min)
Benzene	78.11	80	78	2.994
Octane	114.23	125	43	5.242
2-Pentanone	86.13	102	43	3.265
Pyridine	79.10	115	79	4.125
1-Pentanol	88.15	138	55	4.594

As can be seen in Figure 4.2, our findings showed that RSDs for all compounds were smaller than 2%, up to 300 extraction/injection cycles. Although a slightly decrease in the amount extracted for octane and benzene was observed, the depletion percentage after the 300 extraction is below 5% (Table 4.2), and for applications where the use of a single vial is desired this reduction is insignificant due to the fact that the depletion levels are below 2% for over 250 extraction cycles.

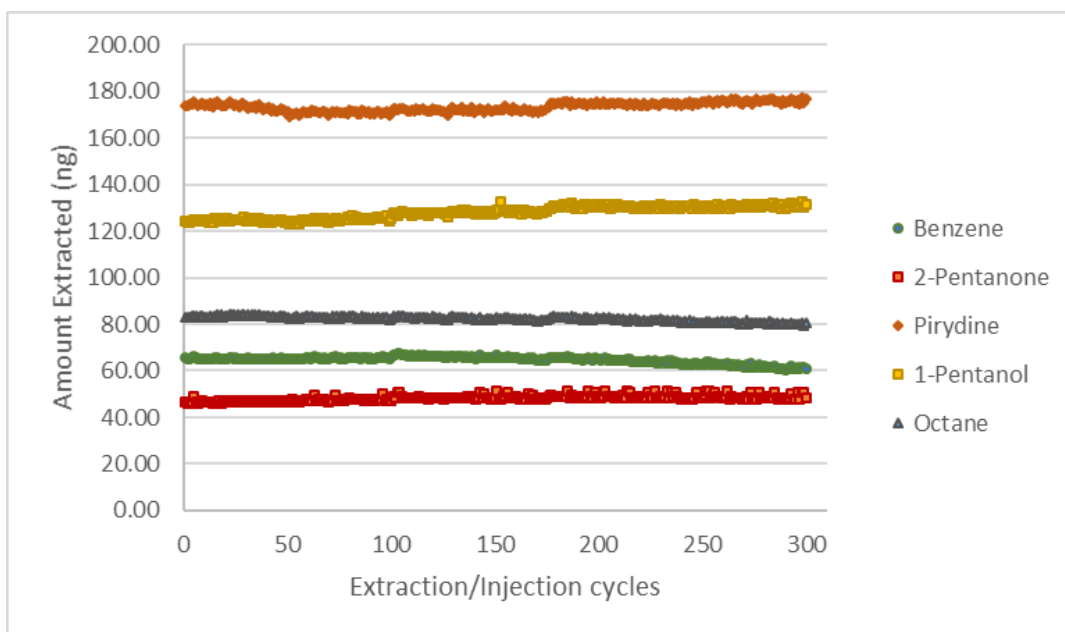


Figure 4.2. Durability of the new gas generator vial using thin film membranes.

It is important to mention that the amount extracted of each standard is similar to the ones previously describe for the vacuum pump oil with SDVB particles⁵⁹ and the silicon diffusion pump oil and polystyrene/divinylbenzene (PS/DVB) particles⁵⁸ ranging from 40 to 180 ng.

Table 4.2. Coefficients of variation and depletion percentages after 300 extraction/injection cycles for the new gas generating vial.

Analyte	RSD %	Depletion %
Benzene	2.62	4.0
Octane	1.50	3.0
2-Pentanone	2.05	0.0
Pyridine	1.06	0.0
1-Pentanol	1.90	0.0

The QC analyses confirmed the reproducibility and reusability of the new gas generating vial as the RSD for these measurements were below 5%; however, normalized values to the initial QC extraction showed that after time there was an increase in the amount extracted in the gas generation vial use for this purpose (Figure 4.3); one explanation for the increase in the amount

extracted over time could be that the vial was initially conditioned for one hour and it was kept in the incubator at 35 °C for the whole run of the 300 extraction (5 days, every QC was performed every 12 hours), which can implied that this type of composite could need more time to equilibrate over time. However, as this type of gas generating vial has been highly tested, a more reasonable explanation could take into account a possible drift in signal in the instrument over time, which was accounted for in the overall evaluation of the reusability of this new vial.⁴¹

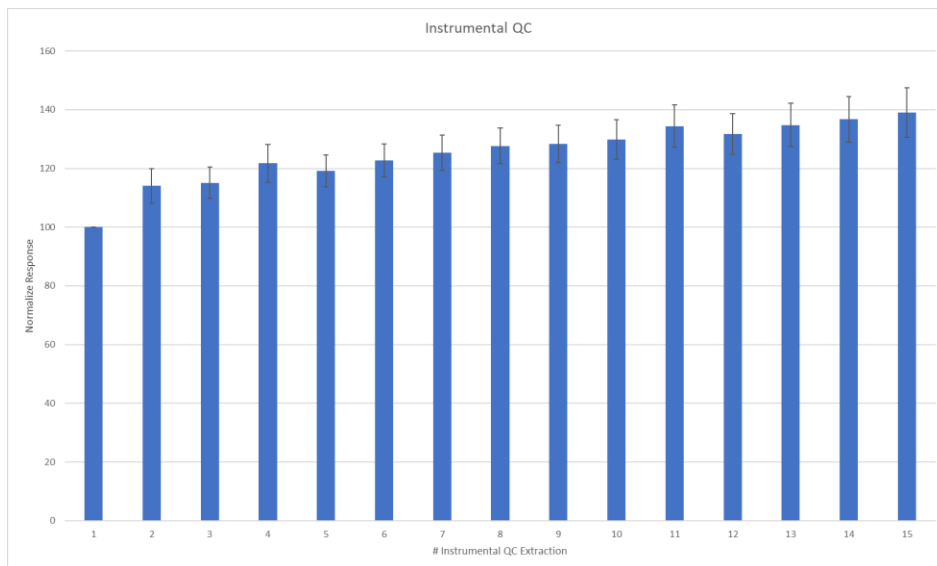


Figure 4.3. Quality control of the PDMS/CAR/DVB fiber after every 25 extractions of the new gas generating vial.

4.4. Conclusions

A new in-vial standard gas system for calibration of SPME in high-throughput applications was presented in this study. The loading technique is fast and reproducible, and the same standard generation vial can be used for more than a 300 analyses, which is essential when processing a large number of samples.

The analyses performed were fully automated by using a CTC PAL3 autosampler system. In addition, due to the compacted and secure appearance of the new gas generating calibration vial, previous issues related to spills or dispersion of the particles in the vial causing differences in the

extraction reusability over time are not a concern anymore. Similarly, the vial can be easily transported, and it is an ideal calibration standard for both bench and field instruments and devices. The evaluation of different types of thin film coatings chemistry is going to be further investigated, as well as, the development of a water standard vial for direct immersion mode using SPME will be an interesting application for this type of thin film membranes in the future.

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