NEAR EAST UNIVERSITY
INSTITUTE OF GRADUATE STUDIES
DEPARTMENT OF ANALYTICAL CHEMISTRY

SUPRAMOLECULAR SOLVENT LIQUID-LIQUID MICROEXTRACTION PRIOR TO HPLC FOR THE DETERMINATION OF SUDAN DYES IN SPICES

M.Sc. THESIS

YAMOUR ALZOUBI

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Approval

We specify that we have read the thesis submitted by Yamour Alzoubi titled "**Supramolecular Solvent Liquid-Liquid Microextraction Prior to HPLC for the Determination of Sudan Dyes in Spices**" and that in our combined opinion it is fully adequate, in scope and in quality, as a thesis for the degree of Master of Science.

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Declaration

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

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Abstract

Supramolecular Solvent Liquid-Liquid Microextraction Prior to HPLC for the Determination of Sudan Dyes in Spices

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Sudan dyes are a class of azo dyes used for a wide range of industrial and scientific purposes (coloring of fuel, staining for microscopy, etc.). Sudan dyes are also desirable as food colorings due to their inexpensive cost and abundant availability. However, due to their carcinogenicity, their use in food is prohibited in the majority of countries, including the EU. Despite this, the Rapid Alert System for Food and Feed of the European Union indicates its presence in numerous commodities. In this study, supramolecular solvent liquid-liquid microextraction (SMS-LLME), produced from tetrahydrofuran/1-Dodecanol (THF:1-DO), was utilized for the extraction of Sudan I, III, and IV dyes from spices prior to their detection by high-performance liquid chromatography. The optimum conditions for SMS-LLME were obtained using 900 µL of (THF:1-DO, 8:1, %v/v) as the extraction solvent, with an extraction time of 1.0 min, ionic strength had no significant effect on the extraction efficiency. Limit of detection (LOD) calculated based on $3S_b/m$ was found in the range of 0.4 to 1.5 µg mL^{-1}. linear calibration graphs were produced with values greater than 0.9950 with %RSD less than 9.09% and %RR ranging from 82.6 to 113.0.

Keywords: High performance liquid chromatography, HPLC, microextraction, SMS, spices, Sudan dyes, supramolecular solvent.

Özet

Baharatlarda Sudan Boyalarının Belirlenmesi için HPLC Öncesi Supramoleküler Çözücü Sıvı-Sıvı Mikroekstraksiyonu

Yamour Alzoubi

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Sudan boyaları, çok çeşitli endüstriyel ve bilimsel amaçlar için (yakıt renklendirme, mikroskopi için boyama, vb.) kullanılan bir azo boya sınıfıdır. Sudan boyaları, ucuz maliyetleri ve bol miktarda bulunabilmeleri nedeniyle gıda boyası olarak da tercih edilmektedir. Ancak, kanserojen olmaları nedeniyle, AB de dahil olmak üzere ülkelerin çoğunda gıdalarda kullanımları yasaklanmıştır. Buna rağmen, Avrupa Birliği'nin Gıda ve Yem için Hızlı Uyarı Sistemi, çok sayıda üründe varlığını göstermektedir. Bu çalışmada, Tetrahidrofuran/1-Dodekanol (THF:1-DO)'den üretilen supramoleküler çözücü sıvı-sıvı mikroekstraksiyon (SMS-LLME), yüksek performanslı sıvı kromatografisi ile tespit edilmeden önce baharatlardan Sudan I, III ve IV boyalarının ekstraksiyonu için kullanılmıştır. SMS-LLME için optimum koşullar, ekstraksiyon çözücüsü olarak 900 µL (THF:1-DO, 8:1, %v/v) kullanılarak, 1.0 dakikalık bir ekstraksiyon süresi ile elde edilmiştir, iyonik kuvvetin ekstraksiyon verimliliği üzerinde önemli bir etkisi olmamıştır. 3Sb/m baz alınarak hesaplanan tespit limiti (LOD) 0,4 ila 1,5 µg mL-1 aralığında bulunmuştur. 0,9950'den büyük değerlerle %RSD %9,09'dan az ve %RR 82,6 ile 113,0 arasında değişen doğrusal kalibrasyon grafikleri üretilmiştir.

Anahtar Kelimeler: Yüksek Performanslı Sıvı Kromatografisi, HPLC, Mikroekstraksiyon, SMS, Baharatlar, Sudan boyaları, Supramoleküler Çözücü.

Table of Contents

Approval....1
Declaration....**Error! Bookmark not defined.**
Acknowledgements....3
Abstract....4
Özet....5
Table of Contents....6
List of Figures....8
List of Tables....9
List of Abbreviations....10
CHAPTER 1....12
Introduction....12
Sudan Dyes....12
Chemical Properties of Sudan I–IV Dyes....13
Toxicology of Sudan I–IV Dyes....15
Metabolism of Sudan Dyes....16
Human Risk....18
Liquid-Phase Microextraction....18
Supramolecular Solvent Microextraction (SMS-LLME)....20
High-Performance Liquid Chromatography (HPLC)....21
Statement of the Problem....22
Aim of this Study....22
Research Questions and Hypothesis....23
CHAPTER 2....24
Literature Review....24
Related Research....24
CHAPTER 3....26
Experimental....26
Instrumentation....26
Reagents and Solutions....26
Apparatus....26
Sampling and Sample Pre-treatment....26

Solid-Liquid Extraction....27
SMS-LLME....27
CHAPTER 4....29
Results and Discussion....29
Selection of Maximum Absorption Wavelength (λ_{max})....29
Optimization of HPLC Conditions....30
Supramolecular Solvent Liquid-Liquid Microextraction (SMS-LLME)....31
Optimization of the Type of Extraction Solvent for SMS-LLME....32
Optimization of the Ratio of SMS-LLME....32
Optimization of the Total Volume of the Combined SMS-LLME....33
Optimization of the Ionic Strength....34
Optimization of Extraction Time....35
Optimum SMS-LLME Conditions....36
Calibration, Quantitation and Figures of Merit....37
Comparison of the proposed SMS-LLME-HPLC with other methods....42
CHAPTER 5....44
Conclusion and Recommendation....44
REFERENCES....45

List of Figures

Figure 1. Different spices. 12
Figure 2. Sudan dyes structures. 14
Figure 3. Chili flakes. 15
Figure 4. Metabolism of Sudan dyes, source (Fonovich, 2013). 17
Figure 5. HPLC instrument. 21
Figure 6. SMS procedure. 28
Figure 7. 3D-Plot of Sudan I, III, and IV. 29
Figure 8. A chromatogram for the three Sudan dyes. 31
Figure 9. Optimization of the ratio of SMS-LLME. 33
Figure 10. Volume of SMS. 34
Figure 11. Optimization of the ionic strength. 35
Figure 12. Optimization of extraction time. 36
Figure 13. External aqueous calibration graph for Sudan dyes. 38

List of Tables

Table 1: Optimum HPLC conditions. 31
Table 2. Optimum SMS-LLME conditions. 36
Table 3. Analytical performance of SMS-LLME-HPLC.....39
Table 4. Relative recoveries of Sudan dyes spices samples.....41
Table 5. Comparison of The Proposed SMS-LLME-HPLC Method with Other Methods for The Determination of Sudan dyes in spices. 43

List of Abbreviations

Abbreviation	Definition
1-DO	1-Dodecanol
ACN	Acetonitrile
AP	Acceptor phase
BW	Band width
CPE	Cloud point extraction
DAD	Diode array detector
DI	Deionized water
DLLME	Dispersive liquid-liquid microextraction
DSSBME	Dual solvent-stir bars microextraction
EF	Enrichment factor
EU	European Union
FSA	Food Standards Agency
GI	Gastrointestinal
HF-LPME	Hollow fiber-based liquid-phase microextraction
HPLC	High-performance liquid chromatography
IARC	International Agency for Research on Cancer
LDR	Linear dynamic range
LLE	Liquid-liquid extraction
LLME	Liquid-liquid microextraction
LOD	Limit of detection
LOQ	Limit of quantitation
MISPE	Molecularly imprinted- solid phase extraction
NP	Normal phase
RCD	Recent Commission Decision
RR	Relative recovery
RR	Relative recoveries
RSD	Relative standard deviation
SCPME	Synergistic cloud point microextraction

Abbreviation	Definition
SFOD-DLLME	Solidification of floating organic drop-dispersive liquid-liquid microextraction
SHS-LLME	Switchable-hydrophilicity solvent liquid-liquid microextraction
SMS	Supramolecular solvent
SMS-LLME	Supramolecular solvent liquid-liquid microextraction
SPE	Solid-phase extraction
THF	Tetrahydrofuran
TRNC	Turkish Republic of Northern Cyprus
USE	Ultrasonic assisted extraction
UV	Ultra-violet
UV-VIS	Ultraviolet-Visible

CHAPTER 1

Introduction

Sudan Dyes

In the food industry, Sudan dyes (I, II, III, and IV) are non-authorized and illegal azo dyes, they are used to improve and maintain the appearance of food products, such as those containing chili, curry, curcuma, and palm oil (Calbiani et al., 2004) (**Figure 1**). In foods, color is very important. Food manufacturers add food dyes, particularly synthetic dyes, to their goods to restore the natural colors lost during processing, to eliminate batch-to-batch variances, and to make products more appealing (Qi et al., 2011). In addition to dietary products, Sudan dyes are widely employed as coloring agents in a variety of chemical industries, including oils, fats, plastics, waxes, gasoline, shoes, printing inks, and spirit varnishing, among others (Dillon et al., 1994).

Figure 1.

Different spices.

The U.K. Food Standards Agency (FSA) provides warnings concerning frozen meat items, spice combinations, and potato chips that contain contaminated chili powder due to contamination with Sudan dyes. Except in certain African and Asian nations, their usage as additives in foods intended for human consumption is prohibited on a global scale. Recent Commission Decision (RCD) (2005) mandates that all food products containing chili, curry, and curcuma as well as palm oil entering any EU state must be certified to be free of Sudan dyes.

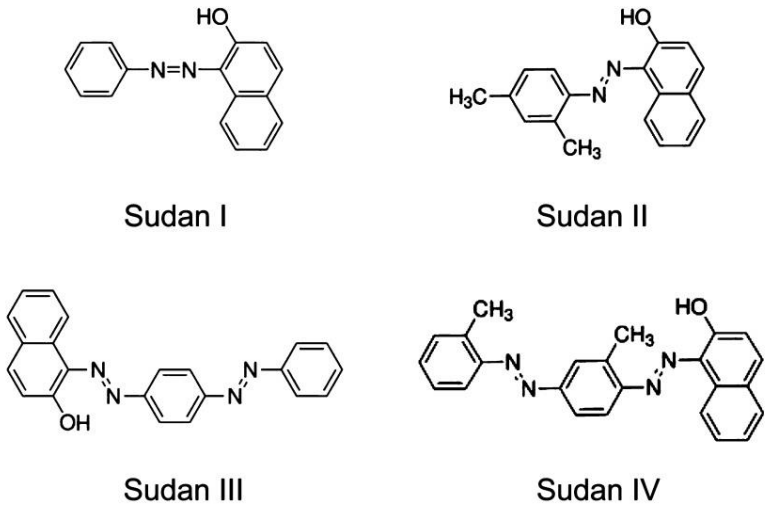
Chemical Properties of Sudan I–IV Dyes

Sudan I is insoluble in water but soluble in a wide variety of chemical solvents. There is no information on the solubility of Sudan II. Sudan III and Sudan IV are ethanol-soluble as shown from their structures in **Figure 2.** Sudan I–IV have solubilities that are extremely comparable.

Sudan I has a *logP* value of 5.86 indicating that it is a highly hydrophobic molecule. Sudan II and Sudan IV still no information about them, however based on their structures, their *logP* values should be comparable or somewhat higher (Abraham et al., 2002).

Figure 2.

Sudan dyes structures.



Sudan dyes are analogues of azobenzene, and the maximum absorbances of Sudan I–IV dyes are 475 nm, 489 nm, 503 nm, and 513-515 nm, respectively, in ascending order. On the other hand, Sigma-Aldrich provides the second absorbances for Sudan II 604 nm and Sudan IV 357 nm. For Sudan I, the first absorbance is 418 nm and the second absorbance is 476 nm.

The acidity of Sudan dyes is low. However, the azo group in the 1-position, which functions as a hydrogen bond acceptor, results in the creation of an intramolecular hydrogen bond between the phenolic OH and the azo group. This extra stabilization of the neutral Sudan molecule accounts for the relatively high *pKa* value of 11.65 for Sudan I and II (Rebane et al., 2010). The *pKa* values of Sudan III and Sudan IV are not predicted to be different. Through the whole pH range, Sudan dyes may thus be considered as neutral compounds.

Toxicology of Sudan I–IV Dyes

Sudan dyes reaction products, have been categorized as "not classifiable as to its carcinogenicity to humans" by the International Agency for Research on Cancer (IARC); thus, they should be removed from food items when their content surpasses 0.5-1.0 mg kg^{-1}. Therefore, there is restricted or insufficient proof in animals and humans, and the EU has therefore blacklisted their use in food (Noguerol-Cal et al., 2008). Sudan dyes are used in cosmetics (Rebane et al., 2010), and tests on animals have demonstrated that isomers of Sudan III produce allergic responses (Okada et al., 1991). In addition, the danger increases with regular intake (Rebane et al., 2010).

Figure 3.

Chili flakes.

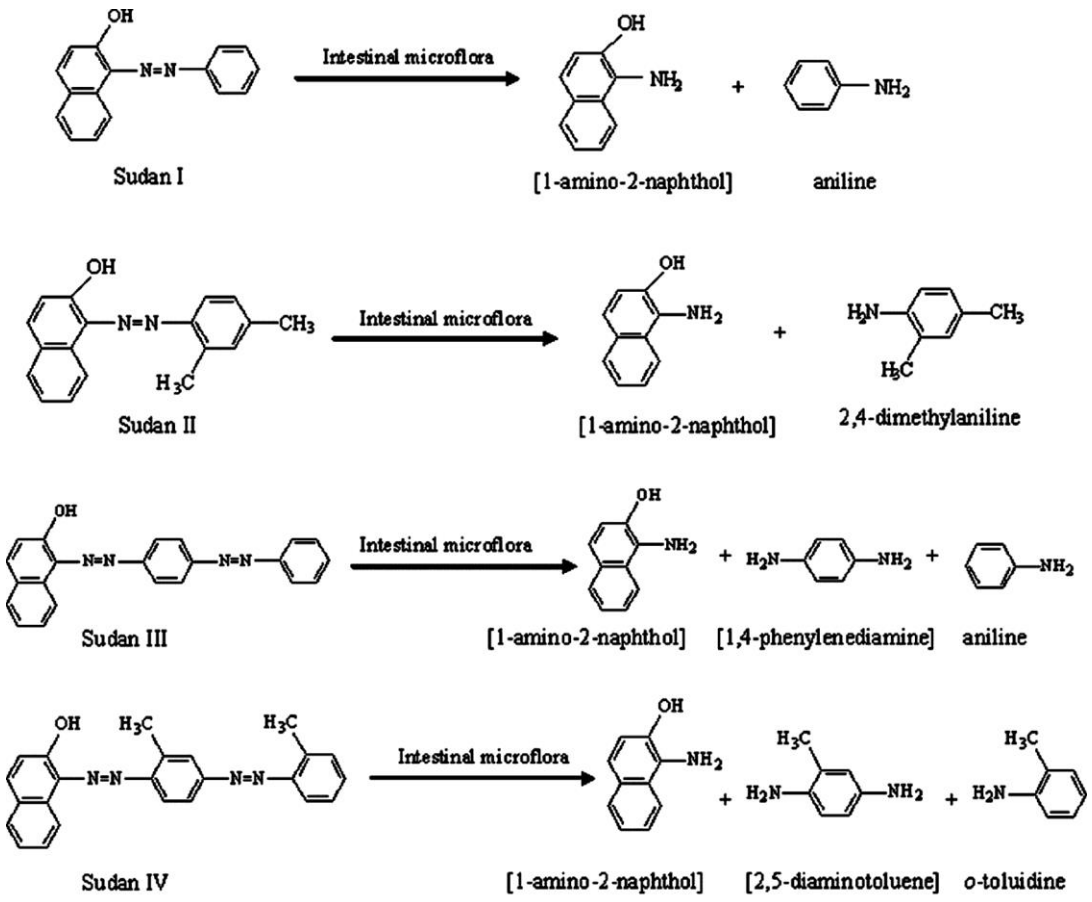
Metabolism of Sudan Dyes

Under anaerobic conditions, microorganisms may work as reductase inhibitors and convert azo colors in the azobenzene to colorless amines. These metabolites may be carcinogenic and harmful (Ramanathan et al., 2009) discovered the enzyme's catalytic residues in order to better comprehend the molecular process behind the biodegradation of Sudan I, II, III, and IV.

According to the produced metabolites, the metabolism of chemical substances in various organs and, preferably, mammalian livers may also be in charge of bioactivation rather than detoxification of parent chemicals. The microflora of the human skin and gastrointestinal (GI) tract are capable of degrading Sudan dyes, resulting in the formation of possibly carcinogenic aromatic compounds (Xu et al., 2007). The metabolism of Sudan I and II is completely in 24–30 hours, however Sudan III and IV were metabolized more slowly, perhaps due to their decreased solubility and availability. Toxic aromatic amines are produced mainly during the degradation of Sudan dyes; Sudan I and Sudan III produce aniline, Sudan II produces 2,4-dimethylaniline, and Sudan IV produces o-toluidine (Fonovich, 2013) (**Figure 4**).

Figure 4.

Metabolism of Sudan dyes, source (Fonovich, 2013).



Recent research (Xu et al., 2010) evaluated the capability of over 30 well-known species of human intestinal bacteria to degrade Sudan dyes. Except for Sudan II, they discovered that the capacity to degrade Sudan dyes is prevalent among human colonic bacteria. The majority of strains examined decreased the amount of colorants. Escherichia coli and Pepto streptococcus magnus were the only bacterial strains unable to eliminate the colorants considerably (Ren et al., 2010).

Human Risk

The wide application of Sudan dyes results in chronic exposure of populations to such chemicals. Sudan III, for example, is still allowed in cosmetics. However, only for items that do not come into touch with the mucous membranes. Sudan II, III, and IV, on the other hand, have been discovered as pollutants in Chinese lipsticks (Fonovich, 2013).

These chemicals can be absorbed via the skin as other substances in a class of items known as "personal care products". According to Nohynek et al. (2010) (Nohynek et al., 2010), There is an urgent need for global organizations to work on safety standards for these products and their ingredients. Sudan III is also utilized to color lipid components in a variety of histological analysis methods, including plant (Aliscioni et al., 2009), animal (Noguerol-Cal et al., 2008), and human evaluations (Patnana et al., 2012). Regular, long-term exposure of humans to allowed substances with low toxicity or that are rapidly eliminated from the body may likewise have adverse effects. Establishing a range for the actual exposure of humans to each of these compounds based on their industrial production, usage, and environmental accumulation would be very valuable. Due to exposure through cosmetic products, contaminated food such as chili powders and turmeric, and other sources (Guha et al., 2011), it is necessary to carry out a risk assessment evaluation approach.

Liquid-Phase Microextraction

As analytical chemistry evolves, sample preparation has the potential to consume up to 80% of the total time of a separation-based analytical procedure, which generally consists of five steps: sampling, sample preparation, separation, detection, and data interpretation. Since then, sample preparation has increased in sophistication in recent years (Chen et al., 2008).

With the advancement of analytical methods and procedures as a result of technology development, including computer, information, and instrumentation, sample pretreatment has fallen behind. In an effort to decrease the number of stages in a technique, and to reduce or eliminate the use of solvents for extraction or ecologically suitable substitute solvents, sample pre-treatment has just lately gained increased attention. Sample preparation affects the vast majority of subsequent assay procedures and is therefore crucial for unambiguous identification, confirmation, and quantification of analytes. Infrequently, a clean sample aids in improving separation and detection, but a sample that has been improperly processed might render the entire experiment incorrect. Using optimally cleaned samples also minimizes the time required to maintain instruments and, consequently, the analysis cost (Chen et al., 2008).

Prior to the determination of any material in complex matrices, sample preparation is always regarded as the barrier of all analytical methods. Due to increased public awareness that environmental toxins pose a health concern, environmental applications are a major factor in the development of several sample preparation techniques. The rising demand for the analysis of foods and natural products has added to the need to enhance sample preparation methods. The government's obligation to improve the quality and level of life of the general public also necessitates the development of more sensitive and reliable methods for monitoring the environment. Recent research has focused on developing miniature extraction procedures that are efficient, cost-effective, and "green". Liquid–liquid microextraction (LLME) with its different modes, such as solidification of floating organic drop-dispersive liquid-liquid microextraction (SFOD-DLLME) (J. Caleb et al., 2021), hollow fiber-based liquid-phase microextraction (HF-LPME) (Pedersen-Bjergaard & Rasmussen, 1999), switchable-hydrophilicity solvent liquid-liquid microextraction (SHS-LLME) (Al-Nidawi et al., 2020), synergistic cloud point microextraction (SCPME) (Al-Nidawi et al., 2022), and dispersive liquid–liquid microextraction (DLLME) (Jude Caleb et al., 2021), among others, has attracted increasing attention as novel sample preparation techniques.

Supramolecular Solvent Microextraction (SMS-LLME)

Supramolecular solvents (SMS-LLME) are a relatively new word (Ballesteros-Gómez et al., 2010) used to refer to nano-structured liquids derived from amphiphiles by the use of a sequential self-assembly process that occurs on the molecular and nanoscales. This procedure yields initial three-dimensional in a second step, aggregates coacervate to form water-immiscible liquids composed of massive supramolecular aggregates. distributed in a continuous phase, which is often water.

Supramolecular solvents offer a unique set of physicochemical features that make them very desirable as organic solvent replacements in analytical extractions. The fundamental inherent features of these solvents are: use of synthetic processes based on self-assembly that are accessible to anyone; the prevalence of amphiphiles in nature and synthetic chemistry, which facilitates their availability; tunable solvent characteristics by modification of the hydrophobic or polar group of the amphiphile; The presence of various polarity sites in the supramolecular aggregates results in good solvation characteristics for a wide range of organic and inorganic substances; non-volatility and non-flammability of supramolecular aggregates that make safer procedures possible.

Because of the non-covalent interaction between SMS-LLME and the self-assembly process that permits their production, these solvents are distinguished from conventional molecular and ionic ones by the term supramolecular. Typically synthesized with tetrahydrofuran (THF) and medium-chained alcohols, reversed micelles are the most prevalent SMS-LLME for analytical purposes. The high concentration of amphiphiles in the solvent enables high enrichment factors in reversed micelle-based supramolecular solvent liquid-liquid microextraction (SMS-LLME) (Ballesteros-Gómez et al., 2010).

High-Performance Liquid Chromatography (HPLC)

In the early 20th century, the development of high-performance liquid chromatography began. In the early 1940s, Martin and Synge started the basic inquiry. In the 1960s, scientists realized that more separation could be achieved by decreasing the inner diameter, reducing the size of the packing materials, and then increasing the flow velocity of the mobile phase, resulting in an increase in pressure. The subsequent decrease in separation time and improvement in resolution necessitated the implementation of high-performance (pressure) liquid chromatography. HPLC instrument is shown in **Figure 5**.

Figure 5.

HPLC instrument.

Due to its exceptional repeatability, high precision, application to a wide range of compounds, and compatibility with a variety of detectors including UV-VIS, fluorescence, electrochemical, and mass spectrometry, HPLC is a highly regarded preparative separation method. In the fields of biology, chemical engineering, food, pharmaceuticals, petrochemical industries, and environmental protection, HPLC has rapidly developed into a versatile, rapid, and highly selective analytical technology.

Six stages constitute a strategy or approach to the creation of this HPLC test:

1. Choosing HPLC methodology;
2. Choosing HPLC column;
3. Choosing beginning experimental conditions;
4. Conducting a preliminary separation;
5. Evaluating the preliminary chromatogram and determining the necessary resolution;
6. Establishing the necessary conditions for the requisite final resolution.

Statement of the Problem

The most challenging and crucial phase in the overall analytical process is sample preparation, especially with complex matrices such as environmental, biological, and food samples. Traditional extraction procedures have a number of disadvantages, including the use of massive volumes of organic solvents, a long analysis time, and significant chemical waste that is dangerous to species and individuals. As a result, different extraction methods must be developed in order to overcome these limitations.

Aim of this Study

This research intends to design an extraction process that is simple, rapid, eco-friendly, and meets the following criteria: preconcentration, minimal use of hazardous solvent, sample cleaning, and effective separation of Sudan dyes without interference from other matrix components.

The objectives as following,

- Developing an SMS-LLME-HPLC method for preconcentration and determination of Sudan I, III, and IV dyes in spices.

- Improving HPLC sensitivity and selectivity by the use of a green, environmentally friendly microextraction technique.

Research Questions and Hypothesis

Considered research questions include the following:

- Will the microextraction method improve the sensitivity of HPLC?
- Will the HPLC method separate the Sudan I, III, and IV dyes in spices?
- Will the microextraction method extract the Sudan I, III, and IV dyes from the sample?

Significance of the Study

SMS-LLME paired with HPLC results in an easy-to-use and rapid methodology for routine quantitative and qualitative analysis that may be used for many types of analytes in various types of matrices, including food, pharmaceutical, environmental, and industrial samples.

CHAPTER 2

Literature Review

Related Research

Sudan dyes are widely utilized in commercial and scientific applications. They have been utilized in scientific study, as well as in paintings and cosmetics (Pan et al., 2011). Due to their bright red–orange color and low cost, Sudan dyes are used illegally as food additives, especially in foods containing chili (such as chilli-, curry-, palm oil, frozen meat products, and mixed spices). However, Sudan I–IV colors are not approved internationally as food additives due of their carcinogenicity (He et al., 2007).

Numerous research on the identification of Sudan dyes in food have been published in the scientific literature, including those employing Solid-Phase Extraction (SPE) cartridges primarily for extracting and cleaning, often followed by acetone extraction (He et al., 2007), (Mazzotti et al., 2008). Due to the fat-soluble nature of Sudan dyes, normal phase SPE is commonly utilized for their removal (He et al., 2007). Molecularly imprinted polymers are yet another sort of solid phase extraction utilized for Sudan I analysis (MISPE) (Puoci et al., 2005). In general, decreased recoveries have been seen when SPE is used for sample cleansing, casting doubt on the efficiency of SPE sample cleansing for Sudan analysis.

Recently, adjustments have been made to the cleanup processes to reduce the quantity of organic solvents (Yu et al., 2008). These can be referred to as liquid phase microextraction (LPME) procedures, which are straightforward, efficient, and economical sample preparation methods. U-shaped hollow fiber–liquid phase microextraction (U-shaped HF-LPME) is a kind of LPME whereby the extraction solvent is contained inside the lumen of a porous hollow fiber with thin walls (Yu

et al., 2008). Dual solvent-stir bars microextraction (DSSBME) is an alternate microextraction technique adapted from HF-LPME to boost extraction efficiency. Another method for separating Sudan dyes is cloud point extraction (CPE), which relies on surfactant-mediated phase separation. It is less expensive, utilizes less hazardous surfactants, and can simultaneously extract and preconcentrate analytes (W. Liu et al., 2007).

Other sample preparation methods, such as ultrasonic assisted extraction (USE) (Tateo & Bononi, 2004), (Ma et al., 2006) and centrifugal sedimentation (Ye et al., 2006)), have also been utilized to improve the extraction efficiency while analyzing Sudan dyes. However, USE is difficult to automate, which is a drawback (E. Mejia et al., 2007).

Different techniques were used for determining Sudan dyes include: Various voltametric techniques (Wang et al., 2015), capillary electrophoresis (Eric Mejia et al., 2007), immunoanalytical (Wang et al., 2009), and chemiluminescence flow injection analysis (Y. Liu et al., 2007). In the presence of additional (complex) food matrix components, the majority of these approaches are designed to achieve selectivity towards Sudan dyes.

CHAPTER 3

Experimental

Instrumentation

An Agilent 1200 series HPLC system (United States) equipped with a quaternary pump, vacuum degasser, autosampler, column oven, DAD detector, and Agilent ChemStation for LC 3D Systems (Rev. B.03.01) software was used to perform chromatographic separations. For the separation, a reversed-phase column (ACE 5 C-18. 3.0 mm ID x 125 mm, 5 m) was utilized. A Mettler Toledo electronic balance was used to accurately weigh solid samples and Sudan dyes standards.

Reagents and Solutions

HPLC-grade acetonitrile, sodium chloride, 1-dodecanol, and tetrahydrofuran were acquired from Sigma-Aldrich (Germany), Deionized water (DI).

Apparatus

Hettich Eba 20 centrifuge (Germany), Sigma-Aldrich Eppendorf micropipettes of varying volumes, and ISOLAB laborgeräte GmbH tips were utilized. Deionized water was filtered using a solvent filtration system (BORU CAM 1000 mL) and Whatman filters (0.45 μm and 0.2 μm) (DI). Ultrasound and water bath were manufactured by ISOLAB laborgeräte GmbH. (Germany). The vortex device was used by Heidolph Reax. A Blomberg refrigerator was used to store samples and standards until analysis.

Sampling and Sample Pre-treatment

Spice samples were purchased from local markets in Cyprus. Samples were stored in dry and cold place.

Solid-Liquid Extraction

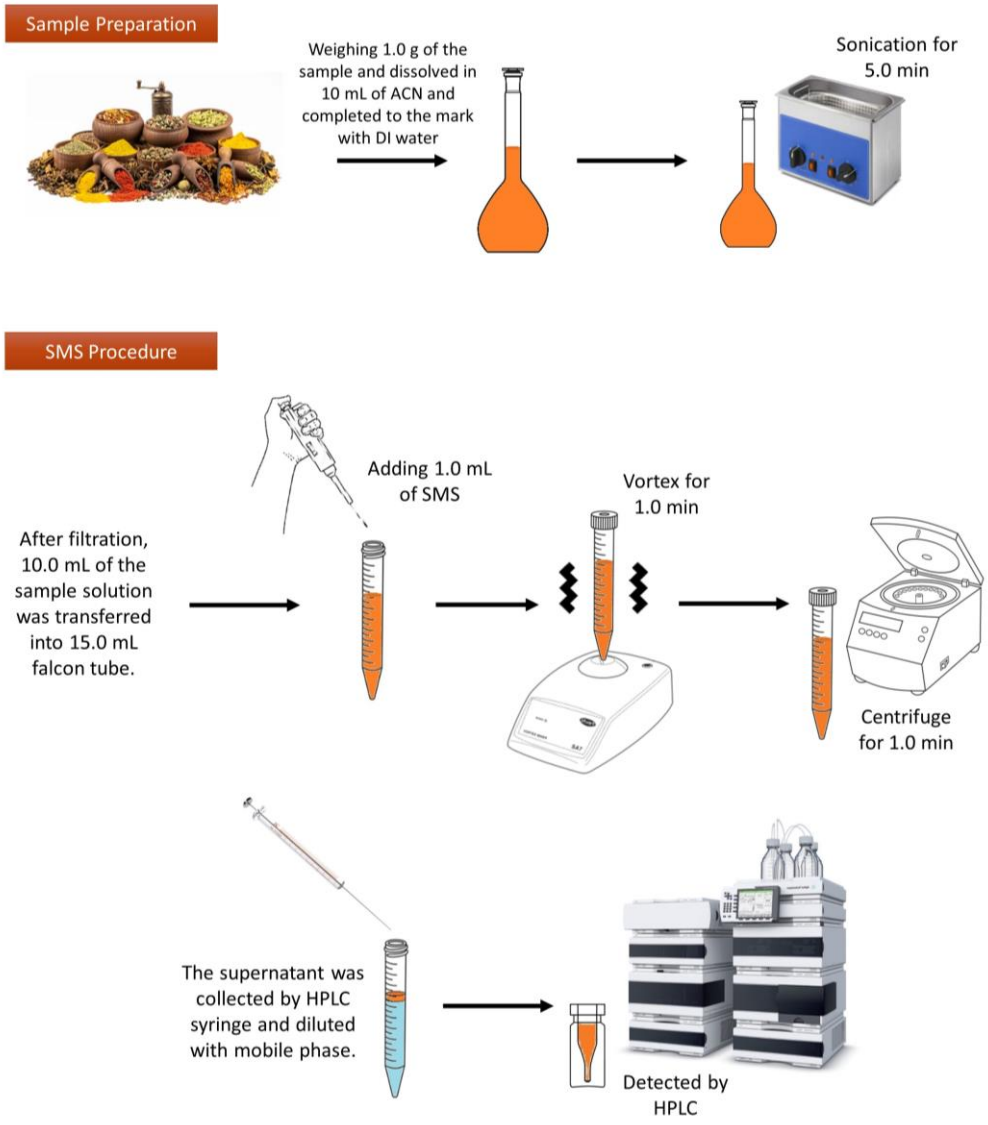
A portion of the fine powder (1.0 ± 0.01 g) was transferred into a 50-mL volumetric flask and 10.0 mL of ACN was added to dissolve the sample by sonication for 5.0 min and then the volume was completed to the mark with DI water.

SMS-LLME

An aliquot of the sample solution (10 mL) was placed into closed capped centrifuged tube and 1.0 mL of SMS (THF:1-DO, 8:1, (%v/v)). The solution was vortexed for 1.0 min and centrifuged for 1.0 min at 6000 rpm. The obtained supernatant was diluted three times with the mobile phase prior to injection to HPLC as shown in **Figure 6**.

Figure 6.

SMS procedure.



CHAPTER 4

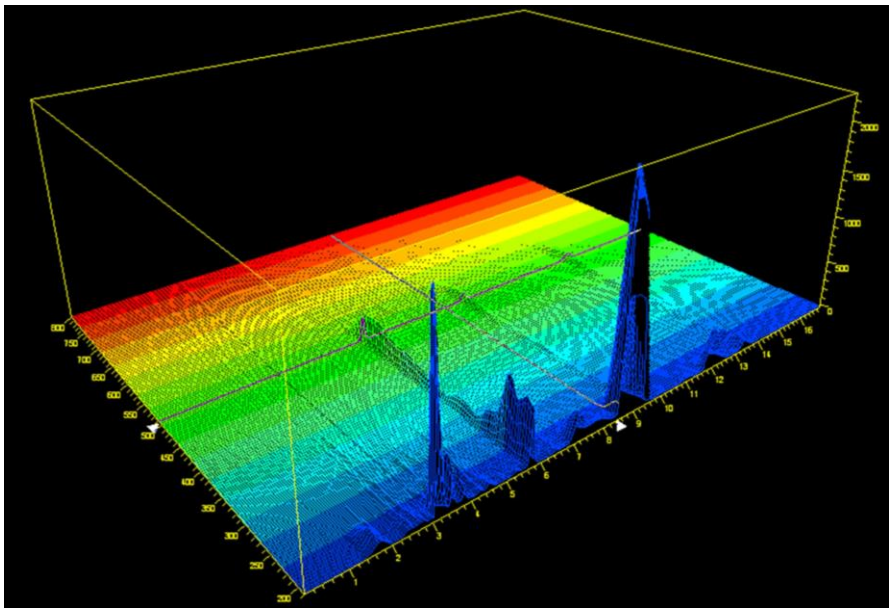
Results and Discussion

Selection of Maximum Absorption Wavelength (λ_{max})

Choosing λ max is a key and crucial stage that aids in reducing errors and enhancing the determination's selectivity and sensitivity. Literature indicates that the maximum absorption of Sudan I, III, and IV are 475 nm, 503 nm, and 513 or 515 nm, respectively. This is the primary choice for λ max. Nonetheless, based on the absorption profile observed in our experiment, 506 nm was selected as the optimum wavelength for the three types of Sudan dyes that used in this study as shown in the 3D-plot property **Figure 7.**

Figure 7.

3D-Plot of Sudan I, III, and IV.



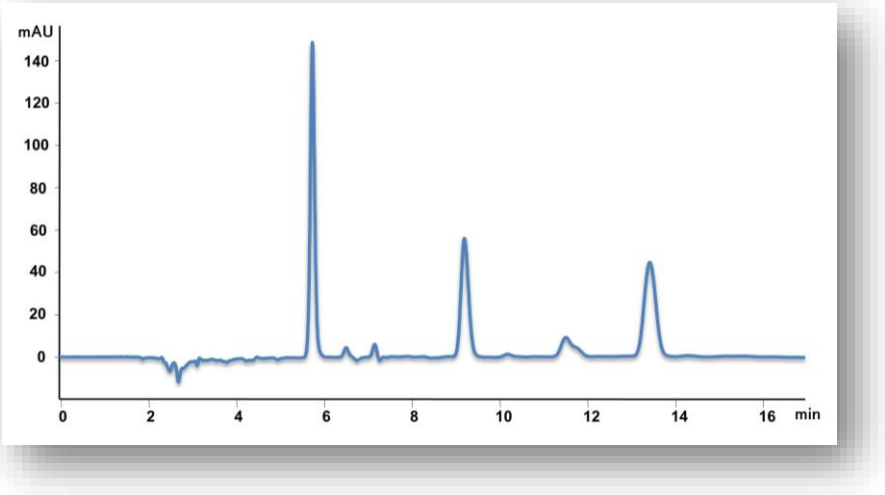
Optimization of HPLC Conditions

The chromatographic system has been developed to achieve excellent selectivity, sensitivity, efficiency, and resolution while reducing band broadening and/or sample component overlap. In order to optimize HPLC conditions, 50 μg mL^{-1} of mixed Sudan dyes were utilized.

Reversed-phase HPLC was selected for this study due to the relative hydrophobicity of the analytes. An ACE 5 C-18 column was selected for separation with a mobile phase composition of ACN/H_2O 60:40 (%v/v), a flow rate of 1.0 mL min^{-1}, a column temperature of 25˚C, and an injection volume of 20 μL as the starting chromatographic conditions. The analysis time was long for the condition, with the last peak eluting after 35 min. To increase the strength of the mobile phase, the amount of ACN was increased to make the mobile phase more hydrophobic since ACN is less polar than water. The optimum mobile phase composition was found to be ACN/H_2O 80:20 (%v/v), which gave an analysis time of approximately 14 min. A good separation with high resolution was obtained for the Sudan I, III, and IV as shown in the chromatogram below (**Figure 8**)

Figure 8.

A chromatogram for the three Sudan dyes.



Chromatogram of 50 µg mL^{-1} of Sudan I, III, and IV, respectively.

Optimum conditions of HPLC are summarized in **Table 1**Error! Reference source not found.**.**

Table 1.

Optimum HPLC conditions.

Physical parameters	Column	ACE 5 C-18. 3.0 mm ID x 125 mm, 5 µm
	Flow Rate	1.0 mL min^{-1}
	Temperature	Ambient (25 °C)
	Detector/wavelength	DAD 506 nm (BW 16).
	Injection volume	20.0 µL
Chemical parameters	Mobile phase	ACN/H_2O 80:20 (%v/v)

Supramolecular Solvent Liquid-Liquid Microextraction (SMS-LLME)

By adjusting extraction parameters, several features of the extraction technique, including its robustness, extraction efficiency, sensitivity, and selectivity, can be

improved. Important SMS-LLME characteristics were examined in detail and optimized with 50 μg mL^{-1} of mixed Sudan dyes.

Optimization of the Type of Extraction Solvent for SMS-LLME

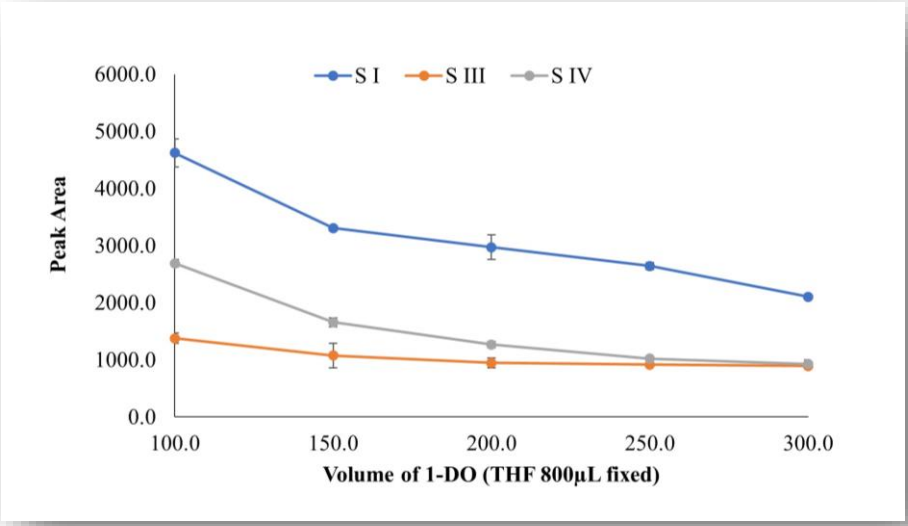
Based on the general principle of SMS-LLME including reversed micelles, the concentration of amphiphiles falls as the alcohol's alkyl chain length increases (Ballesteros-Gómez et al., 2010), SMS-LLME can be formed by combining THF with a long-chain alkyl alcohol. In this study, 1-DO was combined with THF to produce SMS-LLME.

Optimization of the Ratio of SMS-LLME

One of the features of SMS-LLME is that you may vary the volume ratio of the two solvents, their physiochemical characteristics, like polarity. The volume of 1-DO was altered between 100 and 300 μL while the volume of THF was constant (i.e., 800 μL). The peak area was decreasing when the volume of 1-DO was increasing. Therefore, the ratio of THF:1-DO, 8:1, (%v/v) was chosen as optimum for this study as shown in **Figure 9**.

Figure 9.

Optimization of the ratio of SMS-LLME.



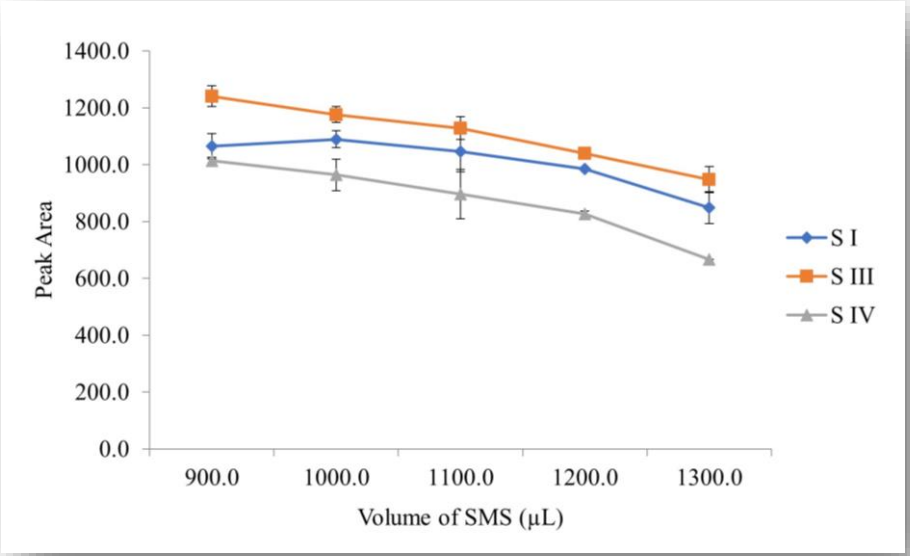
Conditions: Extraction solvent: THF:1-DO; Volume of SMS-LLME: 900 µL; Sample volume: 10.0 mL; Extraction time: 1 min; Centrifugation time: 1 min; HPLC conditions: as mentioned in Table 1**.**

Optimization of the Total Volume of the Combined SMS-LLME

The effect of increasing the volume of the extraction solvent from 900 µL to 1300 µL on extraction efficiency and enrichment factor was investigated (EF). Large volumes of extraction solvent had a negative influence on the peak areas of Sudan dyes, which was a result of the analyte being diluted in the solvent. As the optimum volume for the extraction solvent, 900 µL was selected (**Figure 10**).

Figure 10.

Volume of SMS.



Conditions: Extraction solvent: THF:1-DO; Volume of SMS-LLME: 900 µL; Sample volume: 10.0 mL; Extraction time: 1 min; Centrifugation time: 1 min; HPLC conditions: as mentioned in Table 1.

Optimization of the Ionic Strength

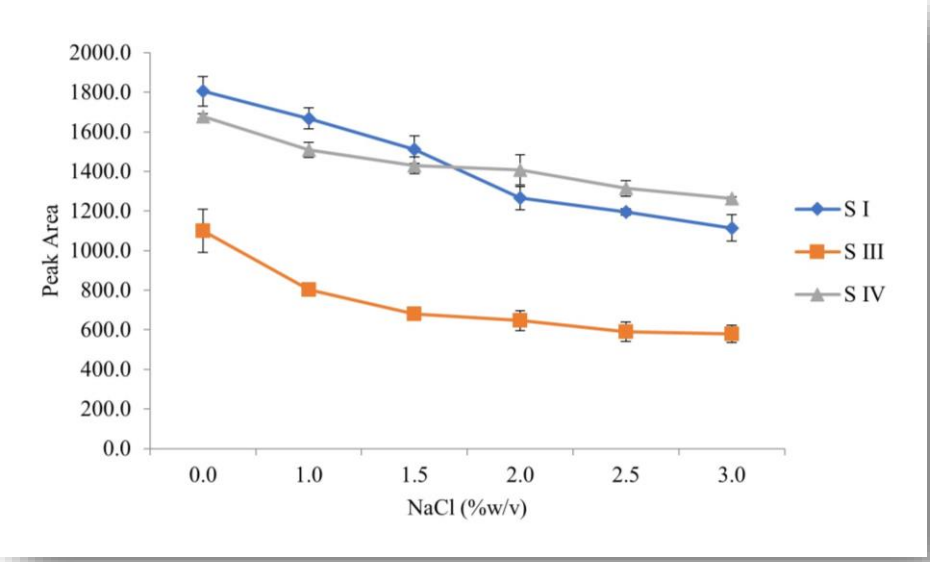
In general, increasing the ionic strength of an aqueous solution by adding a salt increases the solution's polarity, leading to a larger recovery of nonpolar analytes during salting-out. In other words, hydrophobic analytes would become less soluble in the aqueous solution.

Within 0.5% increments, the NaCl concentration in the aqueous solution was increased from 0% to 5% (% w/v). However, no effect or even the opposite effect has been recorded due to other physicochemical features, such as surface tension in addition to the fact that high salt concentrations would result in low analyte recovery due to the extraction solvent's poor miscibility with water as

shown in **Figure 11**. As a result, no salt addition was selected for further experiments.

Figure 11.

Optimization of the ionic strength.



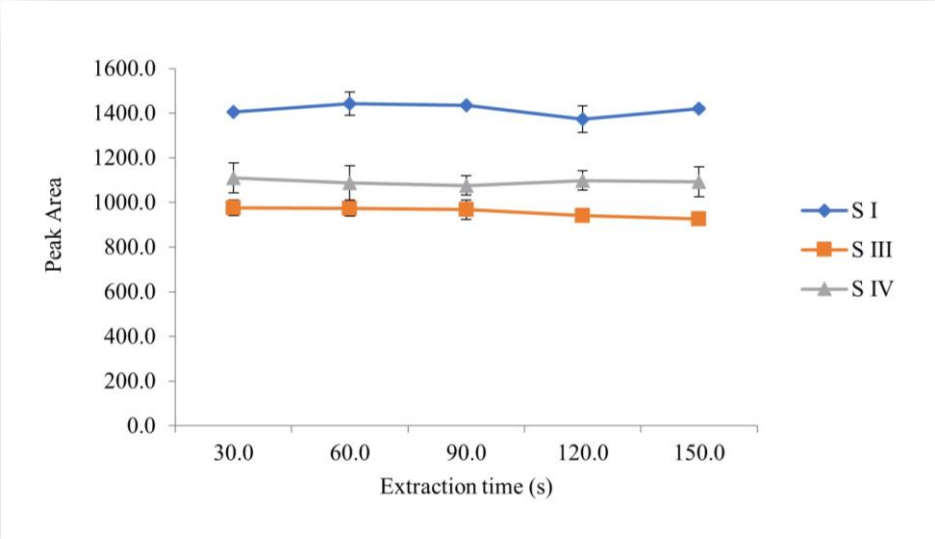
Conditions: Extraction solvent: THF:1-DO; Volume of SMS-LLME: 900 µL; Sample volume: 10.0 mL; Extraction time: 1 min; Centrifugation time: 1 min; HPLC conditions: as mentioned in Table 1.

Optimization of Extraction Time

Vortex mixing can improve extraction by accelerating the equilibrium between analyte and extraction solvent molecules. The extraction (vortex) time was evaluated between 30-150 s. It was shown that extraction effectiveness increased with increasing extraction time up to 60 s before leveling off, indicating that 60 s was sufficient for the construction of SMS-LLME with an enormous surface area for analyte interaction. Therefore, 60 s of vortexing the mixture was determined to be the optimum extraction time (**Figure 12**).

Figure 12.

Optimization of extraction time.



Conditions: Extraction solvent: THF:1-DO; Volume of SMS-LLME: 900 µL; Sample volume: 10.0 mL; Extraction time: 1 min; Centrifugation time: 1 min; HPLC conditions: as mentioned in Table 1**.**

Optimum SMS-LLME Conditions

The SMS-LLME method's optimum conditions are described in **Table 2**.

Table 2.

Optimum SMS-LLME conditions.

Extraction solvent	THF:1-DO
Ratio of the extraction solvent	8:1
Volume of the extraction solvent	1000 µL
Extraction time	60 s
Sample volume	10.0 mL

Calibration, Quantitation and Figures of Merit

Without microextraction, the analytical performance of the proposed SMS-LLME-HPLC technique was evaluated by plotting an external aqueous calibration graph with Sudan I, III, and IV dye standards at concentrations ranging from 5.0 to 100.0 μg mL^{-1} using HPLC (**Figure 13**). Using SMS-LLME, standard-addition calibration graphs were also obtained by spiking food samples with increasing concentrations of Sudan I, III, and IV ranging from 5.0 to 20.0 μg mL^{-1}. **Table 3** presents a summary of the findings. The determination coefficients (R^2) were between 0.9950 and 0.9990, showing excellent linearity. The intra- and inter-day accuracy of the procedure were calculated as %RSD and ranged from 2.23-5.00 and 6.32-9.09, respectively. The LOD was based on $3S_b/m$, where S_b is the standard deviation of the blank signal and m is the slope of the calibration graph ranged from 0.4 to 1.5 μg mL^{-1}, whereas the LOQ was based on $3S_b/m$, where S_b is the standard deviation of the blank signal and m is the slope of the calibration graph ranged from 1.2 to 5.2 μg mL^{-1}. LDR for the aqueous and standard-addition calibration were as high as 100.0 μg mL^{-1} and 20.0 μg mL^{-1}, respectively. Figures of merit are summarized in **Table 3**.

Figure 13.

External aqueous calibration graph for Sudan dyes.

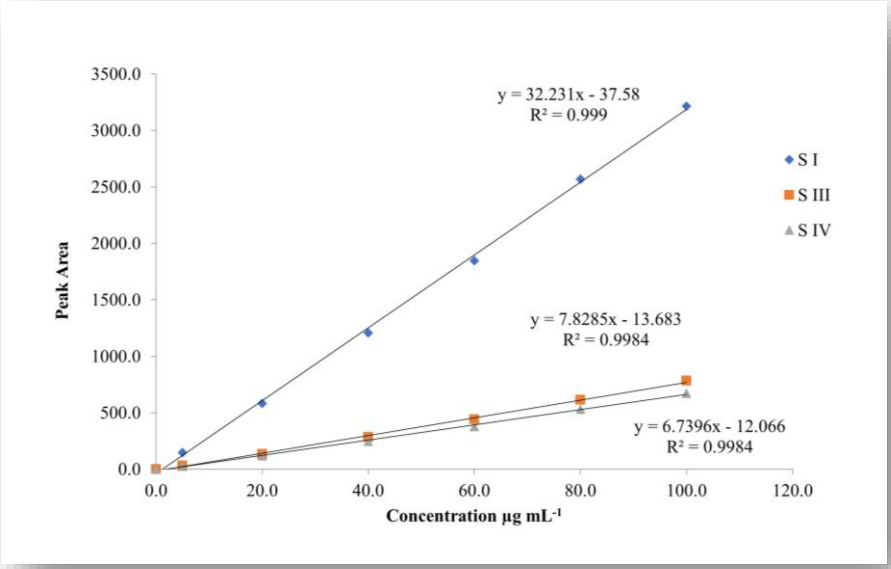


Table 3.

Analytical performance of SMS-LLME-HPLC.

Method	Sample	Analyte	Regression equation[a]	R^2	%RSD[b]		LOD[c]	LOQ[d]	LDR[e]	EF[f]
					Intraday	Interday				
Conventional HPLC	Aq.	Sudan I	$y = 32.23(\pm0.2)x - 37.58(\pm12.9)$	0.9990	4.57	8.44	1.2	4.0	4.0-100.0	-
		Sudan III	$y = 7.8(\pm0.1)x - 13.7(\pm4.0)$	0.9984	4.83	8.93	1.5	5.2	5.2-100.0	-
		Sudan IV	$y = 6.7(\pm0.1)x - 12.6(\pm3.5)$	0.9984	4.10	8.19	1.5	5.2	5.2-100.0	-
SMS-LLME-HPLC	Spice 1	Sudan I	$y = 212.2(\pm3.6)x - 130.3(\pm44.5)$	0.9962	4.35	8.44	0.6	2.1	2.1-20.0	6.6
		Sudan III	$y = 47.9(\pm0.9)x - 21.3(\pm11.1)$	0.9954	3.55	7.64	0.7	2.3	2.3-20.0	6.1
		Sudan IV	$y = 24.3(\pm0.5)x - 9.2(\pm5.9)$	0.9950	3.63	7.72	0.7	2.4	2.4-20.0	3.6
	Spice 2	Sudan I	$y = 50.3(\pm0.5)x - 13.9(\pm6.1)$	0.9987	2.23	6.32	0.4	1.2	1.2-20.0	1.6
		Sudan III	$y = 39.7(\pm0.8)x - 27.4(\pm9.6)$	0.9950	4.83	8.92	0.7	2.4	2.4-20.0	5.1
		Sudan IV	$y = 17.7(\pm0.2)x - 6.1(\pm2.1)$	0.9987	2.45	6.54	0.4	1.2	1.2-20.0	2.6
	Spice 3	Sudan I	$y = 14.0(\pm0.1)x - 0.4(\pm0.3)$	0.9964	3.43	7.52	0.6	2.0	2.0-20.0	4.8
		Sudan III	$y = 156.2(\pm2.6)x - 80.7(\pm31.7)$	0.9964	3.45	7.54	0.6	2.1	2.1-20.0	6.8
		Sudan IV	$y = 49.0(\pm0.9)x - 27.0(\pm11.2)$	0.9954	5.00	9.09	0.7	2.3	2.3-20.0	7.3
	Spice 4	Sudan I	$y = 203.7(\pm4.0)x - 100.1(\pm48.7)$	0.9950	3.58	7.67	0.7	2.4	2.4-20.0	6.3
		Sudan III	$y = 56.3(\pm0.9)x - 24.9(\pm11.5)$	0.9963	4.65	8.74	0.6	2.0	2.0-20.0	7.2
		Sudan IV	$y = 175.8(\pm3.3)x - 117.0(\pm40.4)$	0.9954	4.70	8.79	0.7	2.3	2.3-20.0	26.1

[a]Coefficient of determination

[b]Percentage relative standard deviation ($n = 3$)

[c]Limit of detection µg mL^{-1}

[d]Limit of quantitation µg mL^{-1}

[e]Linear dynamic range µg mL^{-1}

In **Table 3,** regression equations were used to estimate the original concentration (in μg mL^{-1}) of the unspiked samples. By spiking the samples at two concentrations, 5.0 μg mL^{-1} and 15.0 μg mL^{-1}, the percentage relative recoveries (%RR) were calculated and ranged from 82.6% to 113.3%. These recovery numbers helped us adjust for the actual concentration of Sudan dyes in the initial sample. Sudan dye concentrations were below the detection limit (

Table 4).

Table 4.

Relative recoveries of Sudan dyes spices samples.

Sample	Added ($\mu g\ mL^{-1}$)	Found ($\mu g\ mL^{-1}$)			%RR[a]		
		Sudan I	Sudan III	Sudan IV	Sudan I	Sudan III	Sudan IV
Spice 1	-	<LOQ	<LOQ	<LOQ	-	-	-
	5.0	4.42	4.80	5.66	88.4	96.0	113.3
	15.0	15.05	14.37	15.61	100.4	95.8	104.0
Spice 2	-	<LOQ	<LOQ	<LOQ	-	-	-
	5.0	4.74	4.43	4.59	94.8	88.6	91.8
	15.0	14.71	14.85	14.96	98.0	99.0	99.8
Spice 3	-	<LOQ	<LOQ	<LOQ	-	-	-
	5.0	4.74	4.76	4.13	94.9	95.3	82.6
	15.0	14.96	15.07	15.27	99.7	100.4	101.8
Spice 4	-	<LOQ	<LOQ	<LOQ	-	-	-
	5.0	4.90	4.34	4.40	98.0	86.8	87.9
	15.0	14.58	15.49	14.85	99.0	103.3	99.0

[a] Percentage relative recovery.

Comparison of the proposed SMS-LLME-HPLC with other methods

The proposed method SMS-LLME-HPLC was compared with others in the literature which used for the extraction and determination of Sudan dyes in spices in terms of extraction time, type and volume of the extraction solvent, linearity, sensitivity, and precision. From **Table 5**, despite the reality that some of the other methods were superior in terms of sensitivity, the analysis time was fairly long, reaching 40.0 min in dual solvent-stir bars microextraction coupled with high-performance liquid chromatography-ultraviolet/mass spectrometry (DSSBME-HPLC-UV/MS) (Yu et al., 2008) and 57.0 min in U-shaped hollow fiber-liquid-phase microextraction coupled with high-performance liquid chromatography-ultraviolet/mass spectrometry (U-shaped HF-LPME-HPLC-UV/MS) (Yu et al., 2008) which makes the proposed method environmentally friendly. The precision and linearity were acceptable and comparable with the literature.

Table 5.

Comparison of The Proposed SMS-LLME-HPLC Method with Other Methods for The Determination of Sudan dyes in spices.

Extraction method/ technique[a]	Analysis Time (min)	Type of extraction solvent	Volume of extraction solvent (µL)	R^{2}[b]	LOD[c]	%RSD[d]	Ref.
SUPRAS-LLME-LC-DAD	30	decanoic acid/THF	4000	> 0.9960	4.2 µg kg^{-1}	< 7	(Lopez-Jimenez et al., 2010)
DES based UASLME-HPLC	18	Thymol: coumarin	200	> 0.9989	0.35 µg g^{-1}	<3.17	(Ozak & Yilmaz, 2020)
DSSBME-HPLC-UV/MS	57	1-octanol	38	> 0.9945	4.8 µg L^{-1}	< 6.3	(Yu et al., 2008)
U-shaped HF–LPME–HPLC–UV/MS	40	1-octanol	18	> 0.9981	90 ng mL^{-1}	5.7	(Yu et al., 2008)
SMS-LLME-HPLC	7.0	THF:1-Dodecanol	1000	> 0.9950	0.7 µg mL^{-1}	< 9.0	**This study**

[a] SUPRAS-LLME-LC-DAD: Supramolecular solvent-based microextraction and liquid chromatography-photodiode array; DES based UASLME-HPLC: Ultrasound-assisted hydrophobic deep eutectic solvent based solid liquid microextraction with high-performance liquid chromatography; DSSBME-HPLC-UV/MS: Dual solvent-stir bars microextraction coupled with high-performance liquid chromatography-ultraviolet/mass spectrometry; U-shaped HF-LPME-HPLC-UV/MS: U-shaped hollow fiber-liquid-phase microextraction coupled with high-performance liquid chromatography-ultraviolet/mass spectrometry.

[b] Coefficient of determination.

[c] Limit of detection (µg mL^{-1}).

[d] Percentage relative standard deviation.

CHAPTER 5
Conclusion and Recommendation

The desired feature of the extraction method was separation and sample purification of matrix components that interfere with the target analyte. Simultaneously, preconcentration was necessary, particularly for analytes that could be present in low quantities in the sample. In addition, it was desired to reduce the amount of toxic organic solvent.

In this study, supramolecular solvent microextraction (SMS-LLME) was proposed prior to HPLC for the determination of Sudan I, III, and IV dyes in different spice samples. This method satisfied the requirements of green analytical sample preparation, which include environmental friendliness due to the application of smaller volumes of hazardous organic solvents, matrix cleanup, simplicity, cost-effectiveness, short extraction time due to the complete miscibility of the supramolecular solvent with the sample solution, and operability. Analytically satisfactory results were obtained.

On the other hand, for identification, qualitative, and quantitative analysis, RP-HPLC was quick, easy, robust, and sensitive with a 20.0 μL injection volume. The analysis was performed quickly, making it suitable for routine work and efficient.

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