Pontifícia Universidade Católica
do Rio de Janeiro

Beatriz Serrão Monteiro Bastos

Green method for quantification of lavender and sweet orange essential oils in blends by synchronous fluorescence first derivative

Dissertação de Mestrado

Dissertation presented to the Programa de Pós-graduação em Química of PUC-Rio in partial fullfilment of the requirements for the degree of Mestre em Química.

Advisor: Prof. Ricardo Queiroz Aucélio

Co-Advisor: Profª. Rosana Candida Macedo

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This dissertation is dedicated to all who contribute to
and believe in science as a beacon for the progress
of humanity.

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Abstract

Bastos, Beatriz Serrão Monteiro; Aucélio, Ricardo Queiroz (advisor); Macedo, Rosana Candida (Co-advisor). **Green method for quantification of lavender and sweet orange essential oils in blends by synchronous fluorescence first derivative.** Rio de Janeiro, 2024. 105p. Dissertação de mestrado - Departamento de Química, Pontifícia Universidade Católica do Rio de Janeiro.

A simple green analytical method for the quantification of sweet orange and lavender essential oils (EOs) content in blends was developed using synchronous fluorescence first derivative spectra and sample preparation by surfactant-free microemulsions (SFMEs). Excitation and emission wavelengths pairs ($\lambda_{ex}/\lambda_{em}$) were determined for both EOs (at 336/436 nm for sweet orange and at 330/388 nm for lavender). Optimization was conducted to establish the condition to prepare the SFMEs systems: 50 μL of oily phase containing EO diluted in octan-1-ol (1:4, v/v), 2.0 mL of water and propan-1-ol up to 5.0 mL final volume. Different proportions of EO in the oily phase were prepared for analytical curves construction to monitor curve's sensibility. Synchronous fluorescence measurements were performed, and the acquired data were corrected by inner filter effect. Considering the first derivative of the synchronous scanning, data were extracted at 316.8 nm ($\Delta\lambda$ = 100 nm) and 352.0 nm ($\Delta\lambda$ = 58 nm) for sweet orange and lavender EOs, respectively, with determination coefficients above 0.99. Limits of quantification of 8.0 μg mL^{-1} and 65.8 μg mL^{-1} were obtained indicating that 0.46% of sweet orange and 3.69% of lavender EOs can be quantified in mixtures. Simulated blends (25:75%, 50:50% and 75:25%, volume proportions) were also evaluated with recoveries of 71.5 –

128.9% and coefficient of variation between 3.1% – 11.6%. Additionally, the method's greenness was evaluated through three green metrics (Analytical Eco-Scale, GAPI, and AGREE) with aligned results.

Keywords

Sweet orange essential oil; Lavender essential oil; Surfactant-free microemulsion; Synchronous fluorescence first derivative; Analytical quality control; Green metrics.

Resumo

Bastos, Beatriz Serrão Monteiro; Aucélio, Ricardo Queiroz; Macedo, Rosana Candida. **Método verde para quantificação dos óleos essenciais de lavanda e laranja doce em misturas através da primeira derivada da fluorescência sincronizada**. Rio de Janeiro, 2024. 105p. Dissertação de mestrado - Departamento de Química, Pontifícia Universidade Católica do Rio de Janeiro.

Um método analítico simples e verde para a quantificação dos teores de óleos essenciais de laranja doce e lavanda em *blends* foi desenvolvido utilizando a primeira derivada dos espectros de fluorescência sincronizada com preparação de amostras por microemulsões sem surfactante. Os pares de comprimentos de onda de excitação e emissão ($\lambda_{ex}/\lambda_{em}$) foram determinados para ambos os óleos essenciais (336/436 nm para laranja doce e a 330/388 nm para lavanda). A otimização univariada foi conduzida para estabelecer as condições de preparo para os sistemas microemulsionados: 50 μL de fase oleosa contendo óleo essencial diluído em octan-1-ol (1:4, v/v), 2,0 mL de água e propan-1-ol até o volume final de 5,0 mL. Diferentes proporções de óleo essencial na fase oleosa foram preparadas para a construção das curvas analíticas a fim de monitorar a sensibilidade das curvas. Medições de fluorescência síncrona foram realizadas e os dados adquiridos foram corrigidos pelo efeito do filtro interno. Considerando a primeira derivada da varredura síncrona, os dados foram extraídos a 316,8 nm ($\Delta\lambda$ = 100 nm) e 352,0 nm ($\Delta\lambda$ = 58 nm) para os óleos essenciais de laranja doce e lavanda, respectivamente, com coeficientes de determinação acima de 0,99. Limites de quantificação de 8,0 μg mL^{-1} e 65,8 μg mL^{-1}foram obtidos, indicando que 0,46 % de óleo essencial de laranja doce e 3,69 % de óleo essencial de lavanda podem ser quantificados em

misturas dos dois óleos. Misturas simuladas (proporções de volume de 25:75 %, 50:50 % e 75:25 %) também foram avaliadas com recuperações de 71,5 % a 128,9 % e coeficiente de variação entre 3,1 % e 11,6 %. Além disso, o método foi avaliado quanto ao seu impacto ecológico por meio de três métricas verdes (Analytical Eco-Scale, GAPI e AGREE) com resultados concordantes.

Palavras-chave

Óleo essencial de laranja doce, Óleo essencial de lavanda, Microemulsões livres de surfactante, Primeira derivada do espectro de fluorescência síncrona, Controle de qualidade analítico, Métricas verdes.

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List of abbreviations

AGREE	Analytical GREEnness Metric Approach
$A_{\lambda em}$	Absorbance measurements in the emission wavelength
$A_{\lambda exc}$	Absorbance measurements in the excitation wavelength
CV	Coefficient of variation
EO	Essential oil
F_{corr}	Corrected fluorescent signal
F_{obs}	Observed fluorescent signal
GAPI	Green Analytical Procedure Index
GAC	Green analytical chemistry
GC	Gas chromatography
GC×GC-FID	Two-dimensional GC with flame ionization detection
GHS	Globally Harmonized System of Classification and Labeling of Chemicals
IC	Internal conversion
IFE	Inner filter effect
IR	Infrared
MCR-ALS	Multivariate curve resolution and alternating least squares
ME	Microemulsion
MS	Mass spectrometry
NFPA	National Fire Protection Association
NEMI	National Environment Methods Index
O/W	Oil in water
PBT	Persistent, bioaccumulative and toxic
R^2	Determination coefficient
s_b	Blank standard deviation
SDS	Safety Data Sheet
SFME	Surfactant-free microemulsion
ST	Singlet-triplet intersystem crossing
S_0	Ground singlet state
S_1	Lowest excited singlet state

VR	Vibrational relaxation
v/v	Volume proportion
W/O	Water in oil
T_1	Lowest excited triplet state
Δλ	Stokes shift

" A vida é uns deveres que nós trouxemos para fazer em casa.

Quando se vê, já são 6 horas: há tempo…

Quando se vê, já é 6ª-feira…

Quando se vê, passaram 60 anos!

Agora, é tarde demais para ser reprovado…

E se me dessem – um dia – uma outra oportunidade,

eu nem olhava o relógio

seguia sempre, sempre em frente…

E iria jogando pelo caminho a casca dourada e inútil das horas.."

Mário Quintana

1
Introduction

1.1
Contextualization of work

Nowadays, the search for natural products for various purposes is a growing trend. In this sense, the use of essential oils (EOs) and their blends has increased for a variety of applications, including in complementary therapies such as aromatherapy. However, it is important to be aware of the distinct prices of EOs when purchasing their blends, as these commercial products usually do not specify the proportion of each fraction. In this context, fraudulent formulations may contain smaller proportions of the higher-value EO than expected, thereby deceiving consumers. Due to the complex nature of EO, there are few studies in the literature on this topic, underscoring the need for the development of effective analytical methods to quantitative discriminate proportions of different EOs in a blend.

For the present work, a simple analytical method using synchronous fluorescence spectroscopy is proposed, with a focus on quantifying blends of sweet orange and lavender EOs. The method explores specific fluorescence characteristics of each oil. In this case, synchronous fluorescence using the first derivative of the emission spectra was employed to minimize spectral overlap, greatly increasing selectivity [1,2].

However, in this context, the oily nature of the samples must be considered as their high viscosity and opacity hinder direct analysis of EOs, which are

essentially the reasons why fluorescence spectroscopy is not commonly used for this purpose. In order to circumvent this, the EOs were prepared as surfactant free microemulsion (SFME) systems as they are transparent and thermodynamically stable, making two or more immiscible liquids (in this specific case, aqueous and oily phases) stabilized by the addition of short-chain alcohols such as methanol, ethanol, or propan-1-ol, which are used as amphiphilic solvents [3]. Additionally, the formation of micelle-like aggregates restricts fluorophore molecular vibration also protecting them from dynamic quenching, thus decreasing non-radiative processes, consequently enhancing the measured fluorescence signal [4]. Previous studies from the present research group [5] have shown that pseudo-ternary systems with EO and octan-1-ol in the oily phase, together with propan-1-ol and water can substantially increase the fluorescence intensity, lowering sample amount required for analysis.

In addition, the proposed method is in line with most of the green analytical chemistry (GAC) principles, for instance by minimizing both waste and the use of hazardous reagents. The low sample consumption is a significant factor, considering the high value of these products. Additionally, a critical discussion about the method's greenness is accessed in this work through green assessment metrics available in the literature [6–8].

1.2 Dissertation structure

This dissertation is structured into seven chapters. In chapter 1, contextualization of the work and objectives intended with the study are presented.

Chapter 2 contains all the theoretical foundations necessary for the best understanding of the developed work. A brief explanation regarding EO origin, followed by its usage in aromatherapy and quality control regarding blends of these products is presented. Then, the fluorescence phenomenon and its use in spectroscopy is presented, including important related concepts such as conventional and synchronous fluorescent scanning and obtention of derivatives of the spectra. A section regarding explanation of SFME and their differences compared to commonly prepared MEs follows. At the end of the chapter, green chemistry and GAC important concepts are presented, followed by a brief explanation of four metrics presented in the literature to assess greenness of analytical procedures.

Chapter 3 contains all information regarding the experimental procedure, including necessary reagents, materials, instruments and software. Additionally, this chapter presents the detailed procedures initiating with the preparation of the SFME systems, then the univariate optimization to achieve the best condition for analysis. After, the spectrophotometric parameters are detailed followed by data treatment, method validation and green calculators used to assess the method's greenness. Chapter 4 presents all the results regarding the procedures detailed in Chapter 3, including all tables, figures and detailed metrics used to better explain the results. Finally, Chapter 5 presents a concise summary of the results for the dissertation. The subsequent chapters include references used for this work and attachments section.

1.3
Objectives

1.3.1
General objective

Develop an analytical method for quantifying sweet orange and lavender EOs in commercial blends through first derivative synchronized spectrofluorimetry using SFME systems as approach for sample preparation.

1.3.2
Specific objectives

- Conduct preliminary analyses with EOs in SFME using conventional fluorescence scanning to explore the fluorescent characteristics and determine the optimal $\lambda_{ex}/\lambda_{em}$ pair and $\Delta\lambda$ for each EO system.

- Select the best conditions for SFME preparation through univariate optimization experiment aiming low sample consumption, high water content, and small final volume.

- Construct the analytical curves for each EO system and validate a method considering the analytical figures of merit including:, determination coefficient (R^2), limit of detection (LOD) and limit of quantification (LOQ), recovery, coefficient of variation (CV).

- Evaluate the method considering principles of GAC using adequate green assessment metrics available in the literature with critical analysis of the results.

2
Theoretical foundations

2.1
Essential oil's blends usage in aromatherapy

Essential oils (EOs) are transparent liquids produced as secondary metabolites of plants. They are found in oil sacs or oil glands present in various parts of the plant such as peel, leaves, stem, seeds and flowers [9–11]. EOs are usually obtained by steam/dry distillation technique or by mechanical processes in the case of citrus EOs [11]. Produced mainly for plant's defensive mechanisms, these compounds possess properties such as: antifungal, antiviral, antibacterial, anti-inflammatory and antioxidant [9]. These functional characteristics, combined with their unique aromas, make EOs a valuable product in the global market. In fact, the international market of EOs is in a continuous growth with India, United States, France, China and Brazil leading in terms of market value. Besides, Brazil has been the largest exporter of EO by volume, holding this position for over twenty years [12]. This growing trend is explained by the fact that EOs and their combinations are widely used in food, cosmetics, and pharmaceutical industries as well as in the well-known field of aromatherapy [13].

Aromatherapy has been used as an alternative approach for the treatment of various conditions and many of the reported procedures are based on EOs usage. Although the term "aromatherapy" was only introduced in the 1930's [14], historical evidence suggests that such a complementary therapy has found practice for at least 6000 years in India, China, and Egypt [15]. Currently, aromatherapy is

used to improve one's health in physical, mental, and emotional aspects with EOs administered by inhalation, massage, or simple skin application [9]. Studies have shown its effectiveness in reducing perceived stress and depression, improving the quality of sleep and memory, and even controlling pain and chronic diseases [9,15–17]. Many types of EOs and their combinations have been tested for different purposes in aromatherapy.

When mixing different plant extracts, they can interact in an antagonistic, additive/non-interactive, or synergistic way [11,18]. The additive or non-interactive effects indicate that the characteristic of the blend is only the summation of the individual effects, while synergism indicates that the effect of the combination is greater than the sum of the effect produced by each component individually. On the other hand, an antagonistic effect occurs when the combination produces a less favorable characteristic than the sum of the individuals. [11,18]. When producing a mixture, the goal is for the combination to produce, ideally, synergism between the EOs to enhance the benefits associated with their use.

One typical combination of EOs that is available on the market is sweet orange EO (*Citrus sinensis* or *Citrus aurantium dulcis*) [19] and lavender EO (*Lavandula angustifolia*) [19]. Sweet orange EO is known for its antioxidant, cytotoxic, bactericidal, and antimicrobial activities [20,21] and, in aromatherapy, it has great value due to its anxiolytic effect [22–24]. Lavender EO is also extensively studied in literature, also known for its effect against anxiety disorders [25–27]. Studies are found in the literature not only comparing the effects of these two EOs separately [24,28–30], but also in combined formulations [31–35].

In 2009, a study was conducted by Jimbo and collaborators [31], where patients were exposed to orange and lavender EOs and a mixture of the two showed enhancement in self-orientation associated with cognitive function. Years later, in 2017, Muz and Taşcı [32] reported that a one-month period utilization of a mixture of sweet orange and lavender EOs (in a 1:1 proportion) not only enhanced sleep quality of dialysis patients but also reduced fatigue. Other more complex mixtures containing sweet orange and lavender in the composition also have been studied with satisfactory outcomes [33–35].

Currently, it is common to find fragrance houses selling mixtures of EOs, commercially known as blends. These are usually sold with claims of calming, energetic and focus properties that attract consumers. Nevertheless, consumers must always be aware, as with any product, of the origin and quality of the products purchased to avoid health or financial losses.

2.1.2
Quality control for EO's blends

EO's blends are usually sold without specifying the fraction of each EO present in their composition. In the case of blends involving the mixture of sweet orange and lavender EOs it is important to consider the distinct costs associated with each of these EOs. Brazil is the leading player in orange juice exports and, consequently, the largest exporter of orange EO, as it is a by-product of the juice industry [12]. The high production volume of this EO increases its supply, what lowers its selling price compared to other varieties. In this sense, counterfeit formulations may present higher proportions of sweet orange EO and smaller

proportions of the higher value EO (in this case, lavender) than what is expected thus harming consumers.

The composition of EOs is highly complex, potentially containing more than 300 different compounds in a single sample [11]. This complexity increases even more in the case of mixtures, and because of that, quantification of EOs in blends involve separation techniques, coupled with complex mathematical data treatment [36,37], what may limit their use in routine analysis. De Godoy et al. [36] used two-dimensional gas chromatography with flame ionization detection (GC×GC-FID) coupled with multivariate quantitative models (multivariate curve resolution and alternating least squares, MCR-ALS) for quantitative analysis of EOs in perfume. Lebanov et al. [37] also quantified EOs in blends using MCR-ALS algorithms. In this case, data sets were acquired on average mass spectrum obtained from gas chromatography with mass spectrometry (GC-MS). Although both methods have demonstrated success in quantifying EOs in complex mixtures, they require costly equipment maintenance and operators with a high level of expertise. Additionally, the complexity of the mathematical models used demands personnel proficient in chemometrics, which is not always feasible for routine analysis.

Therefore, it is necessary to develop new methods tailored for cost-effective routine analysis, employing simple analytical techniques, and utilizing simplified mathematical treatments. In this sense, luminescence phenomenon, and more specifically fluorescence spectroscopy, emerges as a promising approach to address the complexity of EOs in a straightforward manner as will be further explored. Although luminescence of EOs is not a widely studied topic yet, studies regarding this theme can be found in literature [38–43].

In 2004, Frérot and Decorzant [40] conducted a study to quantify total furocoumarins in citrus EOs that could be implemented in quality control laboratories due to the restriction of 1 ppm of total furocoumarins in cosmetic products. The intrinsic fluorescence of some furocoumarins were attested and for some of them, fluorescence was more sensitive for quantification than UV detection. Later in 2011, Boschi and colleagues [41] conducted a study exploring the fluorescent properties of wild chamomile, lavender, and other EOs species to see their potential topic application in mice. Although only wild chamomile was tested in the mice's skin (with successful detection by fluorescence imaging technique), lavender EO had one of the highest fluorescent emission intensities when tested, showing its potential to also be studied in topical applications for different purposes.

Approximately 30 years ago, Buiarelli et al. [42] pointed out that compounds from the non-volatile fraction of citrus EOs fluoresces with distinct excitation/emission wavelength maxima pairs. More recently, Macedo et al. [43] found that when preparing EOs of this family in SFME systems, the fluorescence of all of them shifted to the same region and presenting maximum fluorescence signal at 336/436 nm excitation/emission wavelength. This finding is valuable for identifying citrus EOs in complex mixtures, including blends with other EOs. Specifically, it suggests the potential for quantifying citrus EOs when blended with other families of EOs, which is the present case in blends of sweet orange and lavender.

2.2

Fluorescence

2.2.1

Fluorescence phenomenon

Photoluminescence is a phenomenon that occurs when photons are emitted by a chemical species after absorption of radiation, and it can be classified as fluorescence or phosphorescence [44,45]. In fluorescence, the radiative transition occurs from lowest excited singlet state (S_1) to the ground state (S_0), being this transition between states of same multiplicity (singlet → singlet). In contrast, phosphorescence occurs involving states of different multiplicity (triplet → singlet) and is much less probable to occur than fluorescence as it depends on the efficient intersystem crossing ($S_1 \rightarrow T_1$) before the radiative emission occurrence in the form of phosphorescence [44,45]. Phosphorescence was not studied in this work so it will not be further discussed.

Besides singlet-triplet intersystem crossing (ST), non-radiative deactivation processes like vibrational relaxation (VR) and internal conversion (IC) can also take place when a species is in an excited state, competing with fluorescence. With a lifetime ranging from 10^{-14} to 10^{-12} seconds, VR occurs as energy disperses as infrared (IR) quanta or kinetic energy due to collisions with other molecules, for example, inducing their decay to the lowest vibrational level in an electronic state [44]. IC takes place as energy dissipates across consecutive electronic levels due to specific conditions, such as small energy gap between the lowest vibrational level of a higher electronic state and the higher vibrational level of a lower electronic state. This phenomenon facilitates energy dissipation without radiative emission,

what competes with fluorescence. A schematic illustration of the mentioned events is presented in Figure 2.1 in a simplified partial Jablonski diagram.

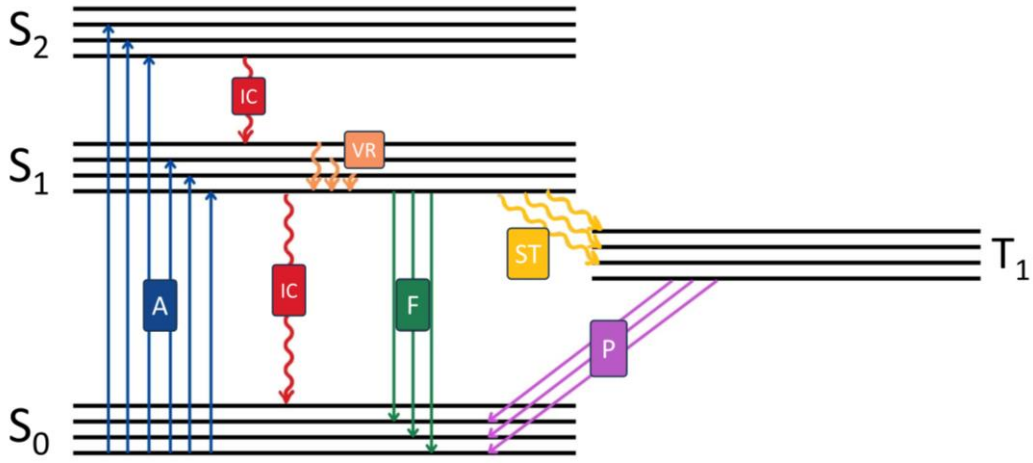

Figure 2.1. Simplified Jablonski diagram representation. In the diagram, "A" stands for absorption, "F" for fluorescence, "P" for phosphorescence, "IC" for internal conversion, "ST" for singlet-triplet intersystem transition and "VR" for vibrational relaxation. Wavy lines represent non-radiative events and straight lines represent radiative events. Colors were employed to simplify visualization.

Species capable of emitting fluorescence are referred to as fluorophores. In the case of molecules, they typically present delocalized π electrons and intrinsic or matrix-induced structural rigidity. However, the molecule's structure is not the only factor that can influence fluorophore's ability to present fluorescence. Additional factors to consider include the solvent in which the fluorophore is dispersed, the system's organization, pH, viscosity, temperature, presence of quenchers, among others [44–46]. In general, factors that difficult the ST intersystem transition or that can restrict the degree of freedom of the molecules, preventing energy loss due to molecule's vibration or collision, enhance fluorescence intensity.

2.2.2
Fluorescence spectroscopy

When fluorescence is employed in analytical purposes to acquire qualitative or quantitative data from a system of interest is referred to as fluorescence spectroscopy or spectrometry. This technique stands out for its selectivity and sensitivity, being capable of detecting analytes at concentrations orders of magnitude lower than absorption spectroscopy [4]. Besides, this technique stands out for its simplicity of operation and speed of analysis [47]. Because of that, fluorescence spectroscopy technique is largely used among analytical chemistry researchers in different quality control fields, including in pharmaceuticals [48,49], food quality assessment [50–52], biochemistry studies [53,54], among others.

Despite its advantages, when using fluorescence spectroscopy for analyte quantification, it is essential to consider that other absorbing species may be present in the sample matrix. If chromophores species absorb at the same excitation or emission wavelengths as the fluorophore of interest, the detected fluorescence intensity will be lower than expected. This phenomenon is known as the inner filter effect (IFE) [45,55]. One way to attenuate it is diluting the sample, but, depending on the preparation of sample or the magnitude of its signal intensity, this is not a viable solution. The simplest method for IFE correction is presented in Equation 1 [55], where F_{corr} corresponds to fluorescent signal intensity after IFE correction, while F_{obs} corresponds to observed fluorescent signal. $A_{\lambda exc}$ and $A_{\lambda em}$ corresponds to absorbance measurements in the excitation and emission wavelength, respectively.

$$F_{corr} = F_{obs} \times 10^{(A_{\lambda exc} + A_{\lambda em})/2} \quad (1)$$

Other forms of IFE correction can be done depending on the sample's nature for more preciseness. In the light of these, to obtain more accurate fluorescence measurements, it is necessary to measure the absorbance within the same range of fluorescence analysis to correct the IFE, especially at high concentrations of the fluorophores, where this effect is even more pronounced.

2.2.2.1

Conventional fluorescence scanning

In conventional fluorescence scanning, excitation spectrum is obtained fixing emission wavelength and varying excitation wavelength in a specific range. The opposite is done to obtain the emission spectrum. When the optimal excitation/emission wavelength pair ($\lambda_{exc}/\lambda_{em}$) is unknown, an "exploratory" search is first performed, varying consecutively emission and excitation in fixed wavelengths until maximum fluorescence intensity is achieved in both excitation and emission spectra.

Besides conventional scanning, where excitation and emission spectra are acquired, there are other ways to obtain qualitative and quantitative information from a sample through its fluorescent characteristics, like the use of polarized; three dimensional; time resolved and, in the present case, synchronous fluorescence spectrometry. Depending on the complexity of the sample and the specific analytical information needed, these methods offer distinct advantages over conventional readings.

2.2.2.2
Synchronous fluorescence spectra

Although fluorescence spectrometry is considered a selective technique, certain limitations persist and must be addressed to obtain accurate analytical data. One such limitation is the potential band's overlap in complex samples [49] as fluorophores with similar excitation or emission spectra may be concomitantly present in the sample. In such cases, a solution that may resolve this issue is the utilization of synchronous fluorescence scanning mode to acquire the spectrum.

Synchronous fluorescence spectroscopy differs from conventional fluorescence scanning in that both excitation and emission monochromators are scanned simultaneously [56], with three distinct types of possible analysis: constant-wavelength, constant energy, and variable-angle luminescence [57,58]. The most popular and simpler between them is constant-wavelength approach, which maintains a fixed $\Delta\lambda$ between λ_{exc} and λ_{em} throughout the analysis. This technique not only narrows the spectral band but can also simplify the emission spectra, and contract the spectral range, what is advantageous for making the fluorescence technique even more selective. Besides, synchronous fluorescence spectroscopy is a rapid and low-cost technique, and depending on the sample matrix, can provide better results than GC-MS and IR spectroscopy [1,2,4].

Introduced by Lloyd [56] in 1971, synchronous fluorescence has been widely used to resolve identification of analytes in complex mixtures. Walash et al. [59] used this technique to overcome spectra overlap of two drugs (metoprolol and felodipine) in a mixture of both, successfully determining both concentrations in a commercial medication. In the food industry, Genis et al. [60], has used the

technique combined with multivariate data analysis to identify different milk origins in dairy products. In his study, synchronous fluorescence has demonstrated advantages over conventional techniques typically employed for this purpose due to its practicality and portability. Arslan et al. [61] also employed this technique with multivariate analysis for cold pressed black cumin seed oil. This study showed that synchronous fluorescence outperformed IR spectroscopy in detecting adulterants. All three studies, and others presented in literature, demonstrate the potential of this approach in routine quality control of complex samples, underscoring the importance of investment in related research efforts.

2.2.2.3
Fluorescence spectra first derivative

When synchronous fluorescence spectroscopy is not sufficient to minimize spectral overlap, it can be combined with derivative technique to provide even more selectivity in the analysis of complex samples [49]. The derivative function is sensitive to sudden changes in a curve, and, as a result, prioritizes regions with abrupt changes over those where the slope changes more gradually [62]. In the case of taking the first derivative of a spectrum, this is evident at the wavelength of maximum intensity, which, in the first derivative, is the point where the function crosses the x-axis. In binary mixtures, regions where the signal intensity of one sample component is zero (or negligible) makes the total signal intensity to be solely attributable to the other component, facilitating its quantification through a calibration curve. These regions are called “zero-cross points” or “annihilation points” [4].

In Figure 2.2B, the red arrows represent two possible zero-cross points of the black curve, where the first one is the nodal point (corresponding to the maximum signal intensity in the spectrum without application of derivative technique) and the second due to absence of signal intensity of the black curve in λ>400nm. In this simulation, both points can be used to quantify the component represented by the blue spectra.

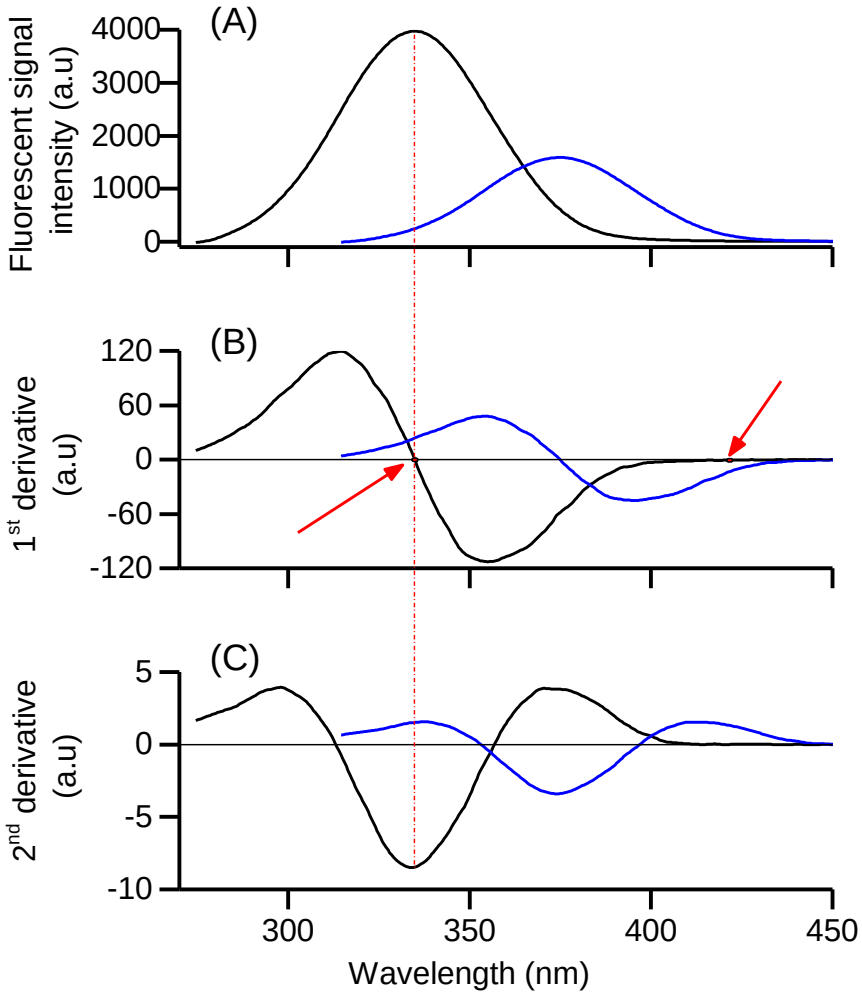

Figure 2.2. Simulated fluorescence signal intensity spectra (A) without derivation, and with (B) 1st derivative and (C) 2nd derivative. Black and blue spectra represent two different samples with fluorescent signal overlap in a mixture. Red arrows indicate the possible zero-cross points of the black spectra. All spectra were smoothed with Savitsky-Golay to diminish signal/noise ratio to facilitate visualization.

Despite its advantages, an important drawback associated with this mathematical approach is that a significant deterioration in the signal-to-noise ratio is observed when taking the first derivative, and this is even more pronounced in

higher orders [4]. In such cases, smoothing algorithms like Savitzsky-Golay [63] are used to attenuate this effect. Furthermore, as can be observed in the figure, the original signal intensity value decreases considerably with the increase of the derivative order, which may impair the sensitivity of the method. Besides that, many researchers were able to apply successfully synchronous fluorescence combined with first and/or second derivatives techniques to quantify analytes in complex mixtures [49,64,65].

When the sensitivity of a method is compromised to achieve greater selectivity, some strategies can be employed to enhance sensitivity. In the case of fluorescence measurements, one effective approach is to use MEs containing the sample of interest to favor radiation process over radiationless ones in deactivation excited state population.

2.3
Surfactant-free microemulsions

Microemulsions (MEs) are systems formed by the mixture of two immiscible fluids (usually water and oily phases) with an amphiphilic solvent that, in the right proportion, spontaneously homogenize itself and generates a thermodynamically stable system [3,66]. The amphipathic solvent, generally a surfactant, reduces interfacial tension by its adsorption on the interface, with its polar head oriented towards the more polar phase (usually aqueous phase) and its less polar tail oriented towards the less polar phase (in this case the oily phase) [66].

The structure of MEs were first elucidated in 1943 by Hoar and Schulman [67] who referred to these systems at the time as “oleophatic hydro-micelles”. It

was only on the following decade that Schulman et al. [68] used the term “microemulsion” for the first time to designate these dispersions (sometimes also termed as solutions due to the long-term stability). Despite it might suggest that MEs are micrometer-scale emulsions, these systems contain particles averaging between 5-100 nm in size. Besides their size and stability, these systems distinguish from traditional emulsions because of their optically isotropic transparency, low viscosity and little energy requirement to be formed [66]. The formation of ME is done by mixing two or three components (in case the system is formed by more than three components) in a certain proportion followed by titration with the final component until transparency is achieved, indicating the spontaneous formation of ME [68].

Nowadays these micelle-like aggregates are known to be divided into three types [69,70]: oil-in-water (O/W), water-in-oil (W/O) and bicontinuous [71] MEs. When the oily phase is in minor quantity and dispersed in the continuous aqueous phase, the system is classified as O/W. Similarly, when the opposite is observed, and droplets of water are dispersed in the oily phase, the system is classified as W/O. An intermediate condition, where both the water and oil phases function simultaneously as the continuous phase, is referred to as bicontinuous MEs. These three types of ME are represented in Figure 2.3.

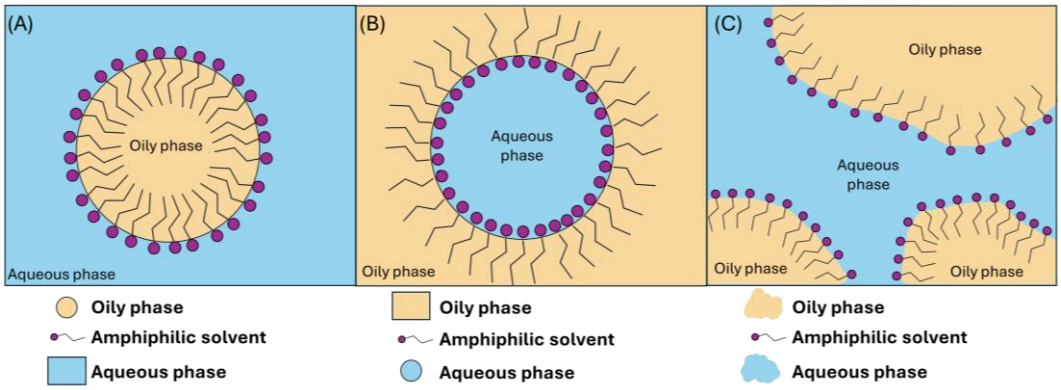

Figure 2.3. Representation of (A) oil in water, (B) water in oil and (C) bicontinuous MEs. Colors were used to facilitate visualization.

Traditionally, MEs are prepared with surfactants because of their strong amphiphilic characteristics compatible with the aqueous and oily phases. Nevertheless, short chain alcohols usually up to three carbons (methanol, ethanol, propan-1-ol and isopropanol) can also be employed for system's stabilization. When these substances are used instead of surfactants as the amphiphilic components, the ME is classified as surfactant-free microemulsion (SFME) [3].

These ternary systems, when formed in absence of surfactants, offer some advantages over traditional MEs. Some to be mentioned include easier separation and purification when used as a reaction medium, lower costs associated with reagents, and reduced risk of toxicity thanks to the absence of surfactants [3]. Additional to reaction medium, SFME can also be used to enhance fluorescence intensity of a fluorophore.

As previously mentioned in this chapter, the organization of the system in which the fluorophores are located directly influences their fluorescent characteristics. When SFME is used to prepare a system containing the fluorophore,

the microenvironment formed can restrict the analyte's degree of freedom and reduce non-radiative processes, like what happens when micellar medium is used [4,72]. Macedo et al. [43] conducted a study demonstrating that the formation of pseudoternary systems with citric EO in a SFME medium (containing water, octan-1-ol and short chain alcohols) was highly effective for analyzing EOs. These micelle-like aggregates significantly increased the fluorescent intensity compared to the EO alone, besides facilitating cuvettes cleaning due to the lower viscosity of SFME. Additionally, studies combining synchronous fluorescence spectroscopy, and the use of organized media have shown a substantial improvement in sensitivity [73–75].

In the light of this, SFME combines the advantages of micelle-like aggregates that constrains the microenvironments and reduces molecular vibrations, thus enhancing fluorescent signal with a greener approach compared to traditional MEs that use surfactants. This dual benefit is extremely relevant in the context of developing more sustainable, eco-friendly methods without compromising efficiency.

2.4
Green chemistry and Green analytical chemistry

The negative consequences of human actions on nature, especially after the Industrial Revolution, have led society to pay more attention to the environment. Within the scientific community, and more specifically in the field of chemistry, the growing awareness about how research and innovations affect the environment is evident. Productions related to the keywords "green chemistry" and "green

analytical chemistry" have had exponential growth over the last 20 years on major search platforms like ScienceDirect, Web of Science, and Scopus (Figure 2.4). Although the absolute number of productions differs, the trend in the graphs is quite similar, showing a rise in the pre-pandemic period followed by slower (yet still positive) growth in the last 3 years. Furthermore, there are indications, especially when analyzing Figure 2.4A and Figure 2.4D, that the number of productions related to these terms will surpass those in 2023 as 2024’s values have already exceeded half of the previous year’s totals. Researchers are increasingly utilizing these concepts to guide their experiments, seeking a greener and more conscious approach. Although many associate the word "green" solely to environmental area, these concepts surpass this field of study, and expands to industry, education and to public scale [76].

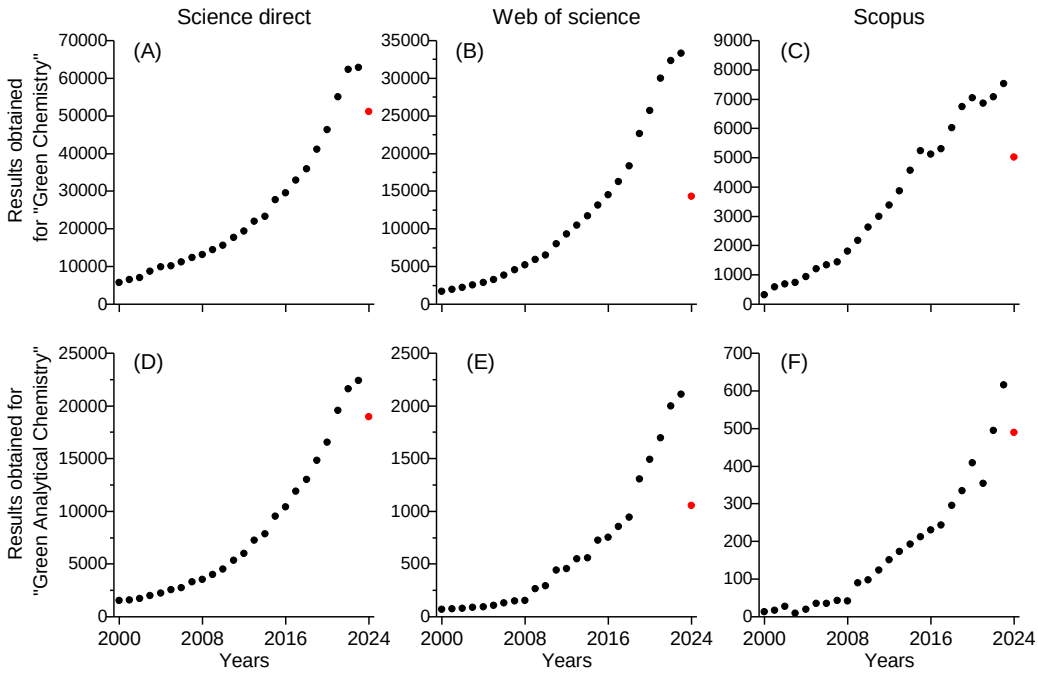

Figure 2.4. Number of productions per year considering the searched terms "Green chemistry" and “Green analytical chemistry” respectively in (A), (D) Science Direct; (B), (E) Web of Science; and (C), (F) Scopus search platforms. The red symbols refer to total results up to the date of data collection (June 29, 2024).

Green Chemistry is a concept created with the aim to eliminate or at least reduce the use of dangerous substances through the sustainable planning of chemical products and processes [76–78]. Introduced in 1998 by Anastas and Warner [78], the twelve principles of green chemistry serve as a guide for achieving these objectives. Some to be mentioned include utilization of catalytic reagents and safer solvents, avoidance of derivatization, prevention of waste and other principles of equal importance. However, the fact that most principles are focused on chemical synthesis and cannot be directly applicable to some situations regarding analytical procedures, led to the development of the concept of GAC.

Emerging from green chemistry, GAC has the challenge to reach an equilibrium point between the quality enhancement of analytical results and their environmental friendliness [79,80]. Based on that, Galuska et al. [79] proposed the 12 principles of GAC, in which only 4 of these were imported from the principles of green chemistry. Those that remained and belong to both concepts are: prevention of waste, use of less hazardous solvent, design for energy efficiency and diminish derivative procedures. The same author also created the mnemonic "SIGNIFICANCE" to simplify the memorization of all principles as seen in Figure 2.5.

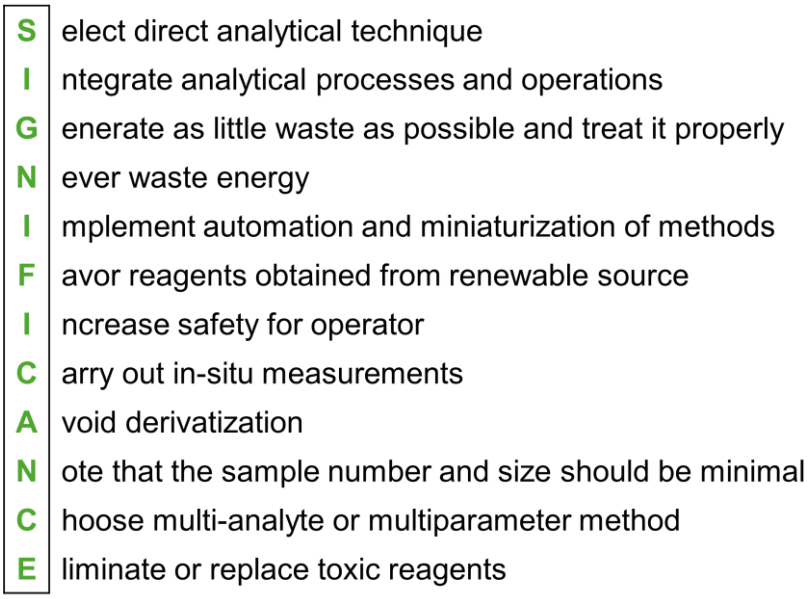

Figure 2.5. Mnemonic "SIGNIFICANCE" for the 12 principles of GAC (adapted from Galuska et al. [79]).

In the last decades, the need to somehow quantify how close the developed method is to an operator/eco-friendly approach has led to the creation of green assessments metrics, also known as "green calculators". These calculators intend to apply the concepts related to green chemistry and/or GAC and generate a score or

a pictogram for the applied method. Besides, the use of these metrics allows researchers to compare different methods available in the literature not only considering their sensitivity and sensibility but also their greenness allowing a deeper critical assessment of the method.

2.4.1 Green assessment metrics for analytical procedures

One of the first tools created for greenness assessment was NEMI (National Environment Methods Index) [81]. This metric consists of an online database that contains information for over 800 methods, however the majority are related to methods for water analysis. To evaluate the greenness profile of the method, a symbol was developed consisting of a circle with four quadrants (Figure 2.6). Each quadrant represents an important criterion regarding the method's safety for humans and its environmental friendliness. If the method meets a criterion, the respective quadrant is colored green and otherwise, it remains blank. These 4 criterias are: 1) Absence of PBT (persistent, bioaccumulative and toxic) chemical. 2) None of the chemicals are listed on D, F, P or U hazardous lists. 3) pH is not < 2 or >12, avoiding corrosive environment during the procedure. 4) Waste generated is not higher than 50 g [82,83].

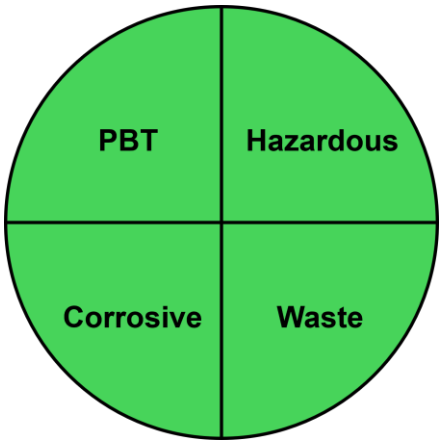

Figure 2.6. Representation of NEMI pictogram. (Adapted from Keith et al. [83]).

At a first glance, the NEMI pictogram is easy to comprehend, however, this metric contains very general information, and the criteria only considers if the threat is below/above a certain value, and this makes this metric not even semi-quantitative [82]. In this sense, other metrics were created to evaluate the method's greenness, including Analytical Eco-Scale, Green Analytical Procedure Index (GAPI) and AGREE (Analytical GREEnness Metric Approach).

In 2012, Galuszka et al. proposed Analytical Eco-Scale [6], a semi-quantitative metric that was based on a previous metric named Eco-Scale [84]. Analytical Eco-Scale proposed the same type of score attribution as Eco-Scale but considering criteria more adequate to analytical methods instead of organic synthesis. The method's evaluation consists of deducting penalty points from a total of 100 for the method's greenness classification. This scale not only considers the quantity of reagents and their toxicity but also their classification according to the Globally Harmonized System of Classification and Labeling of Chemicals (GHS), considering the presence of pictograms and signal words for the assignment of

penalty points. Furthermore, it accounts for occupational hazards and waste treatment.

In practice, to apply analytical eco-scale to the desired method, the user needs to possess Safety Data Sheet (SDS) of all reagents used, as well as their correct amount to calculate penalty points associated with these reagents; gather energy consumption information of all equipment used in the experiment; classify the method as hermetic or non-hermetic; measure the total volume/mass of waste produced and indicate the kind of treatment this waste received. With the summatory of all penalty points and deduction from a total of 100, the user calculates the method's score. According to the authors, it can be classified as excellent (>75), acceptable (50-75) or inadequate (<50) in terms of green analysis. Although generating a score, Analytical Eco-Scale does not generate a pictogram like other calculators and there is no software available to generate the score, so the users need to calculate by themselves, what may lead to mistakes.

Later, in 2018, Płotka-Wasylka [7] developed GAPI, a metric that evaluates the method from sampling to waste treatment at the end of the process, covering the entire analytical procedure. This calculator was developed with the intention of leveraging the advantages of the abovementioned metrics, but also considering GAC aspects that were previously neglected or not so explored by the previous metrics [7]. Similarly to NEMI, GAPI also generates a pictogram (Figure 2.7A), but instead of NEMI's 4 quadrants, this metric includes 15 parameters divided into 5 pentagrams and uses a three-color system (red, yellow, and green) for greenness assessment. The use of three colors allows for a deeper analysis of the parameters and makes the metric more visually informative. This metric does not provide a

score for the tested method, but the user can compare it with other methods by the colors attributed to each one of the 15 parameters. The use of 5 pentagrams divides the parameters into the categories: a stage pre-sample preparation (including collection, preservation, transport and storage), sample preparation (need for extraction or other procedures), classification into direct/indirect method, solvents and their hazard and instrumentation. Besides, in the center pentagram, the presence of a circle indicates if the proposed method is quantitative. Additionally, GAPI classifies the reagents using the National Fire Protection Association (NFPA) system for health and safety hazard evaluation, what also differentiates it from Analytical Eco-Scale. One drawback associated with this metric is that users need to generate the pictogram by hand, which can be time-consuming, especially when used to compare multiple methods. However, the use of the pictogram allows to quickly make a judgement about the greenness of the method.

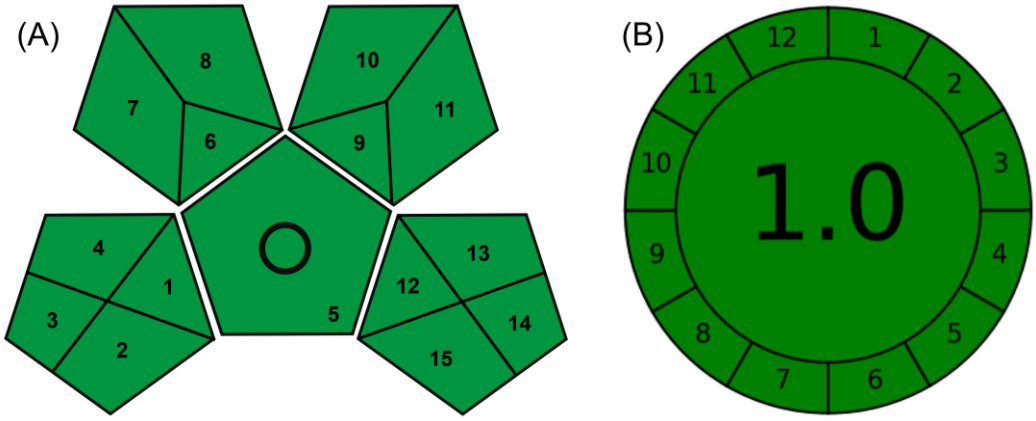

Figure 2.7. Representation of (A) GAPI pictogram [7]. Each number represents a different aspect of environmental and process safety: (1) sample collection, (2) preservation, (3) transport, (4) storage, (5) direct or indirect type of method, (6) scale of extraction, (7) solvents/reagents used for extraction, (8) additional treatments, (9) amount of reagents and solvents, (10) health hazard, (11) safety hazard, (12) energy, (13) occupational hazard, (14) waste amount and (15) waste treatment. The presence of a circle in the middle pentagon indicates that the procedure is suitable for both qualitative and quantitative analysis. (B) AGREE pictogram [8]. Each number in the circle represents a different aspect of environmental and process safety: (1) sample treatment, (2) sample amount, (3) device positioning, (4) sample preparation stages, (5) automation and miniaturization, (6) derivatization, (7) waste, (8) analysis throughput, (9) energy consumption, (10) source of reagents, (11) toxicity, and (12) operator's safety.

Two years after the creation of GAPI, Pena-Pereira et al. developed AGREE [8], a new calculator created as an attempt to overcome gaps and disadvantages regarding previously created metrics. Although AGREE was only published in 2020, it has shown the highest citation growth rate in recent years [85], what may be attributed not only to its ease of access and use but also to its straightforward

comprehensibility. One of the main advantages of this green calculator, if not the primary one, is that the authors have developed a free-downloadable software of this metric, allowing the user to answer a short questionary and obtain automatically the pictogram associated. Besides being very easy to complete, the software also generates a report sheet containing the score associated with each parameter. This metric takes into consideration the 12 principles of GAC (Figure 2.5) for each one of its 12 parameters and results in a user-friendly pictogram of easy comprehension inside and outside the scientific community. The final score is calculated based on results for each parameter and the weight assigned to it by the user. Although this last provides more flexibility to the metric, it can also generate a biased score, as the user can assign a higher weight to the parameter with the greener score, which can complicate a reliable comparison with other methods. In general, this metric is a very rapid, flexible and great tool to help researchers improve the greenness of their methods and compare it to others in literature.

Overall, all presented metrics have their advantages and disadvantages and provide useful information to the user. The best approach is to apply as many metrics as possible to the method and assess if the results are consistent with each other to evaluate if the method is aligned with GAC. However, when applying these metrics users must always exercise critical judgment considering not only the green aspect but also the utility and efficiency of the method in solving a scientific problem or its relevance to a related area. Sometimes, it may be necessary to compromise on specific green aspect in favor of greater sensitivity, for example. The ideal scenario is to strike a balance that combines environmental sustainability with the highest possible efficiency of the method.

3
Material and methods*

3.1
Experimental

3.1.1
Reagents and chemicals

Sweet orange (*Citrus aurantium dulcis*) and lavender (*Lavandula angustifolia*) and thyme *(Thymus vulgaris)* EOs, all commercially available, were obtained from the same Brazilian supplier. For SFME systems preparation, ultrapure water (Millipore, USA), propan-1-ol (Merck, Germany) and octan-1-ol (Sigma-Aldrich, USA) were used.

3.1.2
Instruments, apparatuses and software

Milli-Q gradient A10 ultra-purifier (Millipore, USA) was the source of ultrapure water. Fluorescence measurements were made using a Perkin-Elmer (UK) LS 55 luminescence spectrophotometer. Absorbance measurements were made on a Varian (USA) Cary 100 spectrophotometer. Fluorescence and absorbance spectra were respectively obtained through a FL WinLab software, version 4.00.02 and a Cary WinUV software, version 3.00(182). Optical reflective density filters (Newport, USA) were used for fluorescence attenuation. Data treatment and figures

* A general procedure for quantification of EOs in blends is presented in Attachment B.

were done using OriginLab software (OriginLab Corporation, version 2023b, USA). AGREE software was used for greenness assessment of the method.

3.2
Procedures

3.2.1
SFME preparation

The SFME preparation procedure was adapted from Macedo et al. [43]. Each system consisted of 50.0 μL of an oily phase (EO diluted in octan-1-ol, 1:4 v/v), 2.0 mL of ultrapure water, and propan-1-ol until a final volume of 5.0 mL. The reagents were added in the mentioned order in a volumetric flask, followed by manual agitation for the spontaneous formation of the SFME. This condition was achieved after careful optimization. For method application, SFMEs were prepared with simulated blends of sweet orange and lavender EOs at three different percent proportions (25:75, 50:50, 75:25 v/v). All experiments were performed in three replicates.

3.2.2
Univariate optimization

The univariate optimization was conducted using sweet orange EO since this oil presented higher fluorescence. Preliminary tests were performed from the initial condition: 60 μL of oily phase (EO: octan-1-ol, 1:2 v/v), 2.0 mL of water and adjusting final volume with propan-1-ol until 5.0 mL [43] to obtain the optimum wavelength pair for sweet orange EO and the appropriated $\Delta\lambda$ for synchronous

fluorescence analysis. For univariate optimization, a Stokes shift ($\Delta\lambda = \lambda_{em} - \lambda_{ex}$) of 100 nm was used for obtaining synchronous fluorescence spectra. Three variables were considered for the optimization experiments: volume of oily phase, proportion of EO: octan-1-ol in the oily phase and water amount in the system. Different volumes of oily phase (10, 20, 30, 40, 50, and 60 µL), and different proportions of EO in the oily phase (1:0, 1:1, 1:2, 1:3, 1:4, 1:7 and 1:10 v/v in octan-1-ol) were considered. The condition with the highest fluorescence signal aligned with linear response range was set for water amount optimization (0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 mL). After, compromised condition was set for the following experiments regarding the construction of the analytical curves.

3.2.3
Spectroscopic analysis

Fluorescence spectra were acquired with a 10 nm spectral bandpass for both excitation and emission, and 1500 nm min^{-1} scan rate. Excitation and emission fluorescence spectral ranges were respectively from 250 to 400 nm and from 350 to 550 nm. For synchronous fluorescence spectroscopy analysis, measurements were made from 200 to 500 nm, using $\Delta\lambda$s of 58 and 100 nm. An optical reflective density filter of 10% transmittance (nominal value) was used to attenuate fluorescence intensity. All measurements of sweet orange and lavender were made with the same optical filter. For thyme EO, a filter of 1% transmittance (nominal value) was used. To correct the IFE [55], absorbance spectra (from 200 to 600 nm) were acquired with scan rate of 300 nm min^{-1} and data interval of 0.5 nm.

3.2.4
Data treatment

The fluorescence intensity was adjusted based on the corresponding optical filter calibration values. Then, IFE correction [55] was made by applying the general formula explicit in Equation. 1. The first derivatives of the corrected spectra were calculated and smoothed with Savitzky-Golay algorithm with a window size of 100 data points.

3.2.5
Method validation

For method validation, samples were prepared using sweet orange and lavender EOs, separately, at different volume proportions with octan-1-ol. Conditions named from (A-F) with ratios of 1:4 (A), 1:6 (B), 1:9 (C), 1:19 (D), 1:49 (E) and 1:499 (F) v/v were used. These conditions correspond to concentrations of 1.72 (A), 1.23 (B), 0.86 (C), 0.43 (D), 0.17 (E) and about 0.02 (F) mg mL^{-1} for sweet orange EO and 1.78 (A), 1.27 (B), 0.89 (C), 0.45 (D), 0.18 (E) and about 0.02 (F) mg mL^{-1} for lavender EO, as summarized in Table 3.1.

Table 3.1. Codes for different volume proportions of sweet orange EO:octan-1-ol and lavender EO:octan-1-ol and their respective concentration in mg mL^{-1}.

Code	**EO: octan-1-ol proportion (v/v)**	**Sweet orange EO (mg mL^{-1})**	**Lavender EO (mg mL^{-1})**
A	1:4	1.72	1.78
B	1:6	1.23	1.27
C	1:9	0.86	0.89
D	1:19	0.43	0.45
E	1:49	0.17	0.19
F	1:499	0.02	0.02

Synchronous fluorescence spectroscopy analyses were performed for each sample. First derivative spectra of the synchronous spectra (after treatment) were obtained. To construct the analytical curves, data were collected at zero cross points for each EO (316.8 nm for sweet orange EO and 352.0 nm for lavender EO) that are the wavelengths that spectral contribution of one EO is minimum when measuring fluorescence from the other.

Analytical figures of merit were calculated including limit of detection (LOD) (Equation 2) and limit of quantification (LOQ) (Equation 3) where s_b corresponds to blank standard deviation (n=7) and m corresponds to the analytical curve sensitivity. Linear response was evaluated through the R^2 with residual constructed for homoscedasticity determination. These parameters were also obtained for the analytical curves in which the IFE was not corrected to compare the results. Simulated blends at three percent proportions of sweet orange and

lavender EO (25:75, 50:50, 75:25 v/v) were used to assess the effectiveness of the proposed method.

$$LOD = 3\times s_b/m \quad (2)$$

$$LOQ = 10\times s_b/m \quad (3)$$

3.2.6 Greenness Assessment

Method's greenness was evaluated through three metrics available in the literature, including Analytical Eco-Scale [6], GAPI [7] and AGREE [8]. Analytical Eco-Scale table of penalty points and GAPI pictogram were created by hand, while for AGREE, the software related to this metric was downloaded and the pictogram was automatically obtained. A critical discussion about the method's application in these three green calculators was accessed as well as advantages and disadvantages regarding each one.

4
Results and discussion

4.1
Development of the method

4.1.1
Univariate optimization

Pseudo-ternary systems involving the oily phase (EOs in octan-1-ol), water and propan-1-ol have proven to be suitable for dispersing and stabilizing EO in order to conduct spectrofluorometric analysis. These systems (in this case SFME) require lower amounts of EO, which is advantageous considering the high aggregated value of such samples. Besides, MEs are microenvironments where less polar fluorophores allocate, providing large fluorescence enhancement factors [5,43].

Preliminary studies were conducted to establish the best characteristic wavelength pairs ($\lambda_{exc}/\lambda_{em}$) for each type of EO in SFMEs. Experimental condition, previously described by Macedo et al. [43], was used as a starting point. In this case, SFME was prepared with 60 µL of oily phase (EO:octan-1-ol, 1:2 v/v) 2.0 mL of water and propan-1-ol until final volume of 5.0 mL. Under these conditions, the maximum excitation (λ_{ex}) / emission (λ_{em}) wavelength pairs were 336/436 nm for sweet orange EO and 330/388 nm for lavender EO, showing a clear spectral difference between these two EOs. As a result, the $\Delta\lambda$ were 100 nm and 58 nm respectively (Figure 4.1).

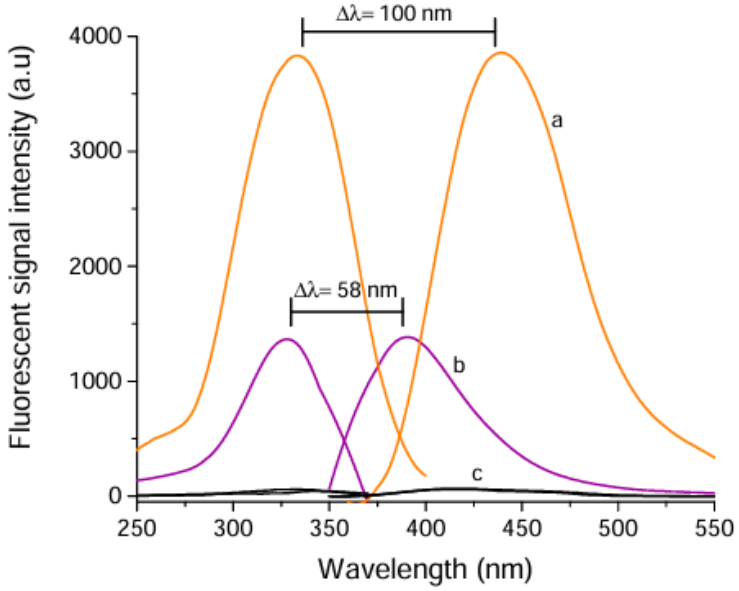

Figure 4.1. Fluorescence excitation and emission spectra obtained for sweet orange and lavender EOs in SFME media. SFME consisting of 60 μL of oily phase (EO : octan-1-ol, 1:2 v/v), 2.0 mL of ultrapure water and final volume adjusted with propan-1-ol until 5.0 mL. The spectra represent (a) sweet orange EO, (b) lavender EO and (c) blank measurements.

After determining the characteristic Δλ value, optimization was conducted using sweet orange EO. This EO was chosen not only because it exhibited a higher fluorescence response, but also because the obtention process of citric EOs contributes to its lower water solubility compared to EOs from other species [43]. This makes it advantageous to use it for optimization, as the microemulsion formation region for orange EO would likely encompass that of lavender, whereas the opposite might not happen.

For the optimization, the oily phase volume and dilution factor of EO in SFME were first evaluated, with water amount fixed at 2.0 mL. Volumes of 10, 20,

30, 40, 50 and 60 µL of the oily phase were tested. For each condition, different volume proportions of EO and octan-1-ol in the oily phase were used: 1:0 (100% EO), 1:1 (50% EO), 1:2 (33.3% EO), 1:3 (25% EO) 1:4 (20% EO), 1:7 (12.5% EO) and 1:10 (9.1% EO). These samples were analyzed by fluorescence spectroscopy to establish robust conditions for SFME formation, taking into consideration the response in terms of signal intensity. Synchronous fluorescence spectra intensities were measured at $\Delta\lambda$ = 100 nm and the intensities recorded in function of volume of the oily phase and percentage of EO in the oily phase can be seen in Figure 4.2.

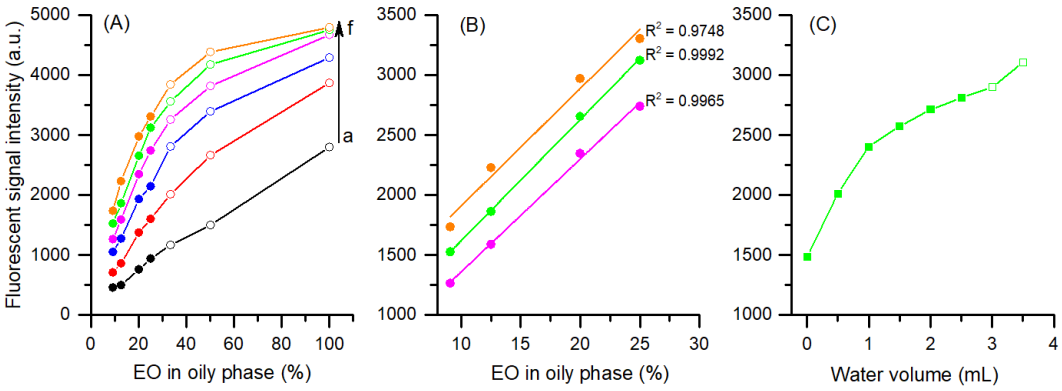

Figure 4.2. Optimization experiments for establishing the formation conditions of SFME systems from sweet orange EO: (A) Fluorescent signal intensity obtained for systems with six volumes of oily phase (a) 10, (b) 20, (c) 30, (d) 40, (e) 50 and (f) 60 µL using different dilutions of EO in oily phase (1:0 or 100% EO; 1:1 or 50% EO; 1:2 or 33.3% EO, 1:3 or 25% EO; 1:4 or 20% EO; 1:7 or 12.5% EO and 1:10 or 9.1% EO). (B) Zoom at the linear range obtained for (d-f) with the R^2 highlighted. 2.0 mL of ultrapure water and propan-1-ol were used for SFME systems formation. (C) Evaluation of water amount effect (0.0 to 3.5 mL) for the condition with 50.0 µL of oily phase and 20% of EO in oily phase (1:4 proportion). Unfilled markers indicate systems with non-satisfactory visual aspect or linearity.

Measurements were made in synchronous scan mode (200 to 500 nm, $\Delta\lambda$ = 100 nm).

From Figure 4.2, it becomes evident that an increase in EO within a constant volume of oily phase leads to a rise in fluorescence intensity. However, beyond 25% of EO (equivalent to a 1:3 proportion), the linear response of the systems begins to decrease, particularly noticeable in larger volumes of the oily phase. Given the objective of maximizing fluorescence intensity to enhance method's sensitivity while reducing volume of oily phase, due to the high intrinsic value of the EOs sample, the 50 μL oily phase volume was chosen. This decision was based on the observation that the linear response of the last four data points surpassed that of 60 μL and the intensity was higher than that achieved with 40 μL (Figure 4.2B).

Additionally, the 1:4 proportion of EO: octan-1-ol was chosen as a compromise condition to ensure it was within a linear working range. Evaluating the water content under this condition (50.0 μL of oily phase, EO:octan-1-ol, 1:4 v/v), turbidity was visually observed in the system upon adding a volume of 3.5 mL of water (Figure 4.3H). To ensure that the system would be within a robust range for the formation of SFME, it was chosen to maintain a volume of 2.0 mL of water. In summary, compromise condition used for the subsequent experiments was set at 2.0 mL of water and 50.0 μL of oily phase (EO:octan-1-ol, 1:4 v/v).

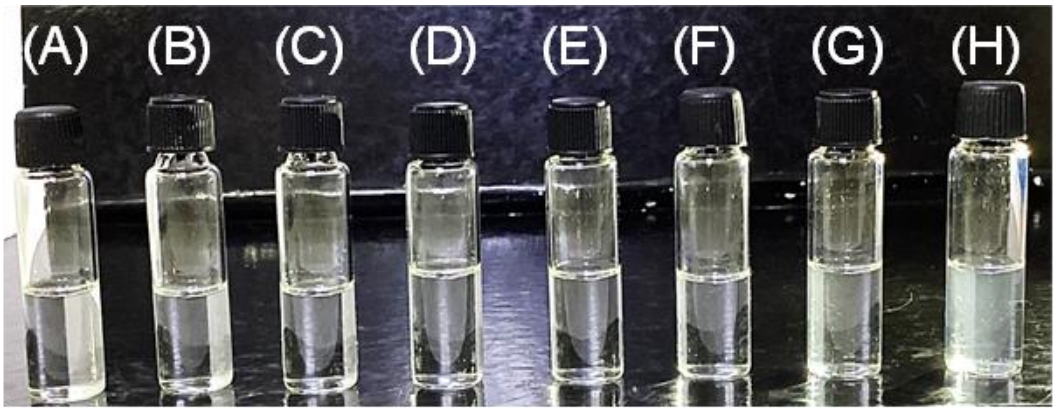

Figure 4.3. SFME systems from sweet orange EO containing different amounts of water: (A) 0.0 (B) 0.5 (C) 1.0 (D) 1.5 (E) 2.0 (F) 2.5 (G) 3.0 and (H) 3.5 mL. For each sample, 50.0 µL of oily phase with EO diluted in octan-1-ol (1:4 v/v), and propan-1-ol up to 5.0 mL were used.

4.1.2
Spectrofluorimetric analysis and data treatment

With a compromise condition set, analytical curves were constructed regarding sweet orange and lavender EOs. Synchronous fluorescence spectral data were first organized in spreadsheets for optical filter correction, then absorbance data (Figure 4.4) was used for IFE correction (Equation 1). The obtained smoothed spectra for sweet orange and lavender EOs at $\Delta\lambda$ = 100 nm and $\Delta\lambda$ = 58 nm are present in Figure 4.5A-B, respectively. The first derivative of the corrected spectra in the two cases were also obtained (Figure 4.5C-D) and smoothed (Savitzky Golay, 100 data points) for the analytical curve construction.

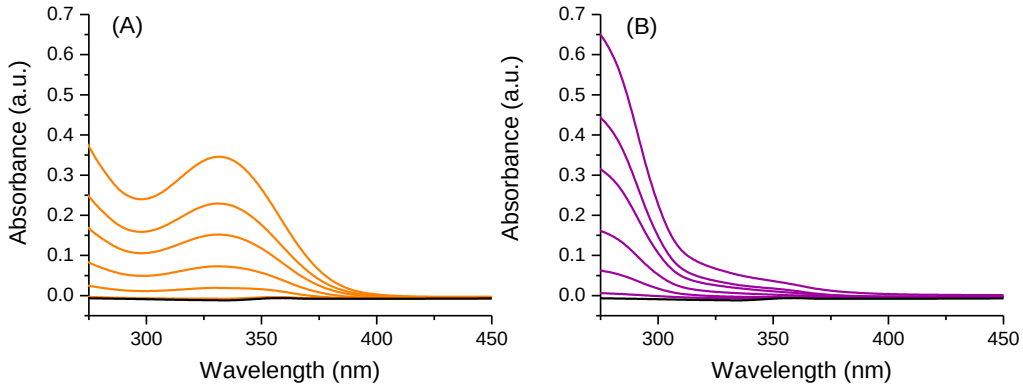

Figure 4.4. Absorbance measurements of SFME prepared with EO: octan-1-ol of 1:4, 1:6, 1:9, 1:19, 1:49, 1:499, v/v for (A) sweet orange and (B) lavender. SFME: 50.0 μL of oily phase (containing EO and octan-1-ol), 2.0 mL of water and propan-1-ol up to 5.0 mL final volume.

To select the optimal wavelength for data extraction, the concept of "zero cross point" was considered, where the total intensity at a certain wavelength is determined by only one component of the binary mixture, as the other has a negligible value regardless of concentration [4]. This occurs because the derivative of the maximum fluorescence intensity intersects the x-axis, and the total intensity is solely attributable to the other component. Using this concept, at $\Delta\lambda$ = 100 nm, the wavelength of 316.8 nm was considered the zero-cross point for lavender and used to construct the sweet orange curve. Similarly, at $\Delta\lambda$ = 58 nm, the selected wavelength was 352 nm for lavender curve construction as highlighted in Figure 4.5C-D. The use of this mathematical is adequate for discriminating signals associated with different compounds when analyzing first derivative spectra [4].

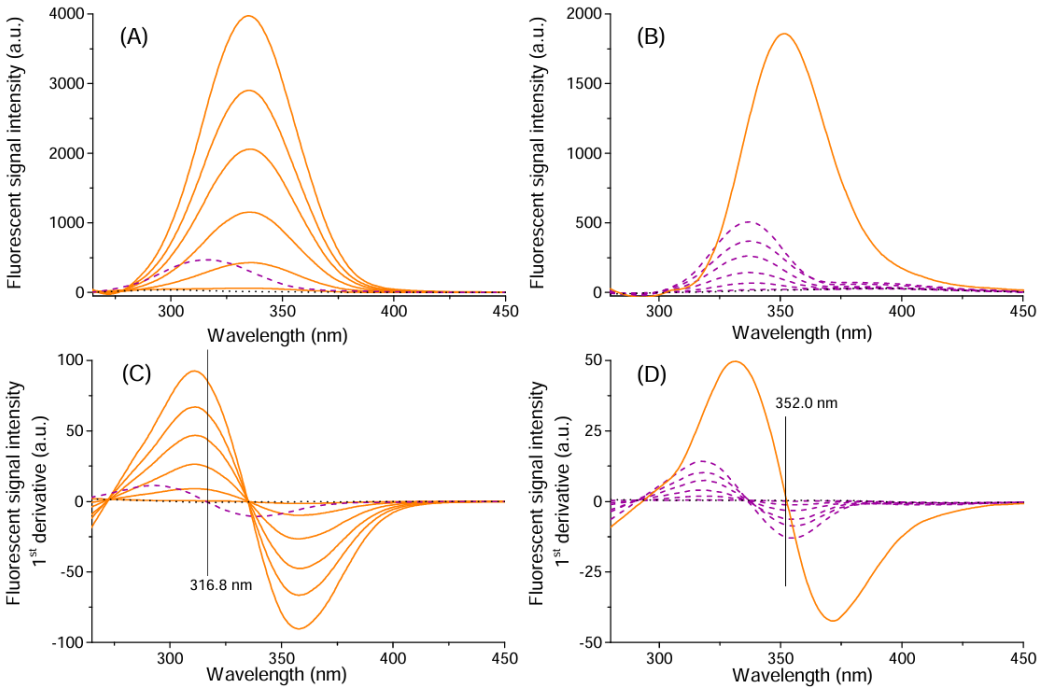

Figure 4.5. Synchronous fluorescent signal intensity at (A) Δλ = 100 nm and (B) Δλ = 58 m for sweet orange and lavender EO samples in SFME systems after IFE correction. Fluorescence intensity first derivative at (C) Δλ = 100 nm and (D) Δλ = 58 nm respectively for sweet orange and lavender EO samples in SFME systems after IFE correction. At Δλ = 100 nm, proportions of sweet orange EO: octan-1-ol of 1:4, 1:6, 1:9, 1:19, 1:49, 1:499, v/v and proportion of lavender EO: octan-1-ol, 1:4, v/v were used. At Δλ = 58 nm, proportion of sweet orange: octan-1-ol, 1:4, v/v and proportions of lavender EO: octan-1-ol of 1:4, 1:6, 1:9, 1:19, 1:49, 1:499, v/v were used. Zero cross point is highlighted in the spectra for (C) lavender EO and (D) sweet orange. SFME: 50.0 μL of oily phase (containing EO and octan-1-ol), 2.0 mL of water and propan-1-ol up to 5.0 mL final volume. Solid line represents sweet orange EO measurements, dashed line represents lavender EO measurements and dotted line represents blank.

4.1.3
Method validation

In order to confirm the effectiveness of the IFE correction upon curve linear response, sweet orange and lavender EO analytical curves were constructed with the same parameters but without the use of correction formula for fluorescence intensity. The R^2 of 0.9690 and 0.9960 were obtained for sweet orange EO and for lavender, respectively. Analytical curves and the associated residual plots are presented in Figure 4.6A-C for sweet orange and in Figure 4.7A-C for lavender EO. In the case of sweet orange, it is observed that there is an absence of random distribution of residuals around the x-axis. Besides, Figure 4.6C also indicates that most of the residuals are not concentrated around the zero point, as expected, showing the importance of IFE correction. However, for lavender EO the same is not observed, as apparently there is a more homogeneous distribution of residuals and linearity does not seem compromised.

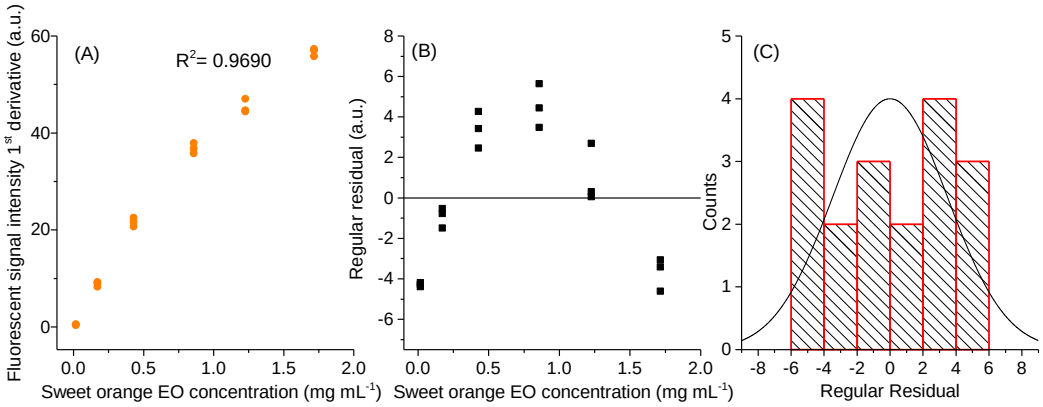

Figure 4.6. (A) Analytical curve based on data obtained for sweet orange analysis without IFE correction. (B). Residual plot and (C) histogram of residual plot are presented to evaluate the linearity of the analytical curve. For analytical curve construction, the fluorescent signal intensities were collected at λ = 316.8 nm from the first derivative of the synchronous scanning spectrum (Δλ = 100 nm).

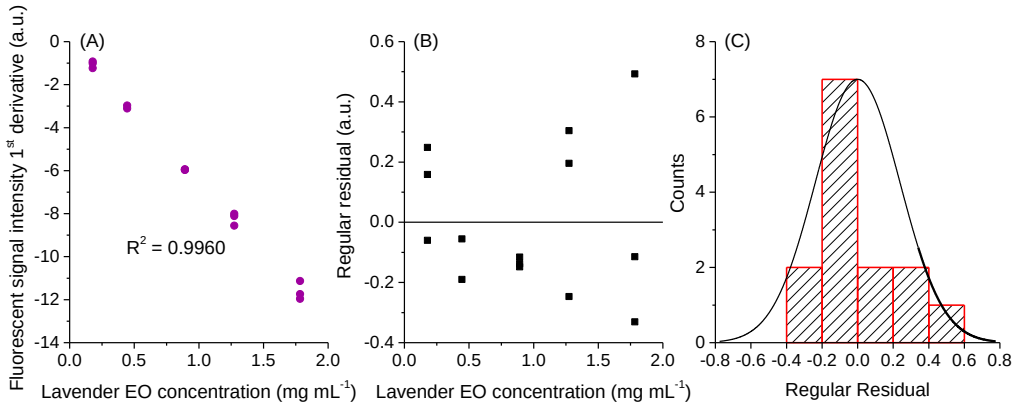

Figure 4.7. (A) Analytical curve based on data obtained for lavender analysis without IFE correction. (B). Residual plot and (C) histogram of residual plot are presented to evaluate the linearity of the analytical curve. For analytical curve construction, the fluorescent signal intensities were collected at λ = 352.0 nm from the first derivative of the synchronous scanning spectrum (Δλ = 58 nm).

Considering the IFE correction, the same procedure was performed not only for sweet orange EO but also for lavender EO. Upon ordinary least square linear regression, R^2 of 0.9967 and 0.9948 were obtained for sweet orange EO and lavender EO analytical curves, respectively. A histogram of the residual plot for each group data was also obtained, and in both cases, a random distribution of points around zero was obtained (Figure 4.8A-B). The R^2 increases substantially and the residual plots for sweet orange EO analytical curve indicates a more adjusted and selective response when compared to Figure 4.6A, where the IFE was not corrected.

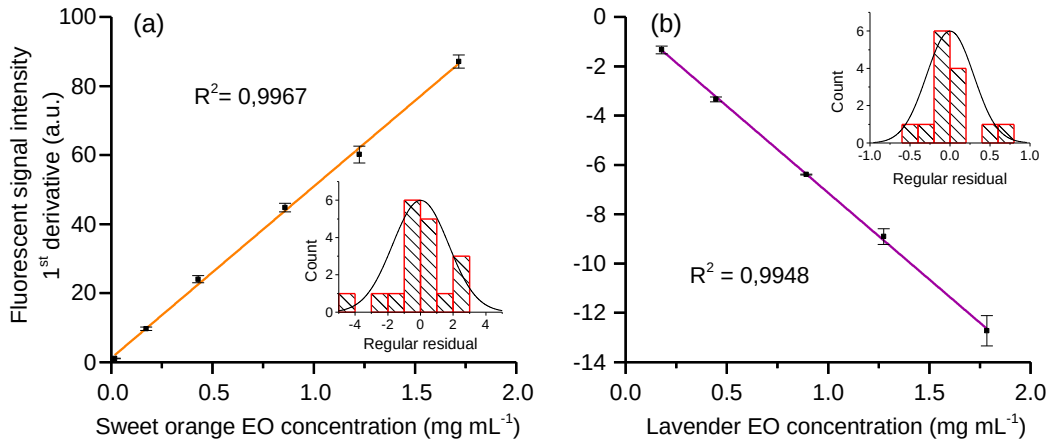

Figure 4.8. Analytical curves (ordinary least square linear regression) based on results obtained for (a) sweet orange and (b) lavender EOs analysis. For analytical curve construction, the fluorescence intensities were collected at λ = 316.8 and 352.0 nm from the first derivative of the synchronous scanning spectrum (Δλ = 100 and 58 nm for sweet orange and lavender EOs, respectively). Concentration varying from (A) to (F) and from (A) to (E) were used to construct sweet orange and lavender EO analytical curves, respectively. Residual plots are presented for each graph.

In the case of lavender EO, little difference was observed between R^2 value and histogram of residuals comparing data before and after IFE correction. This is explained as the lavender EO has a lower fluorescence intensity compared to orange, it can be inferred that the concentration of radiation-absorbing species in this EO is also lower. This fact makes the IFE less relevant, as it is concentration-dependent, and if there are fewer absorbing species, the filtering effect of these species will not be as significant in affecting linearity as in the case where they are present in higher quantities.

This can also be explained by Equation 1. In this equation, it is noted that the observed fluorescence (F_{obs}) deviates from the "real" fluorescence (F_{corr}) by a factor of 10 raised to an exponent that contains the absorbance values. When the value of this exponent is small or zero, the observed and corrected fluorescence are equal or very close, which does not result in a loss of linearity. However, as the absorbance intensity increases, as in the case of sweet orange EO, the exponent also increases, and the observed fluorescence signal deviates further from the expected fluorescence value by a factor of 10 raised to this exponent. Therefore, this effect is much more relevant in the case of sweet orange EO than in lavender EO. However, since the correction is made for sweet orange EO, the same procedure was also applied to lavender, even though it is not necessary, as can be seen by comparing Figures 4.7 and 4.8b.

The analytical figures of merit were obtained for the group data corrected for IFE. The slope and intercept values, as well as their intervals considering 95% of confidence were calculated for each EO (Table 4.1). Besides, LOD and LOQ were calculated based on Equation 2 and Equation 3. The LOQ shows the

possibility of quantifying a minimum of 0.46% of sweet orange and 3.7% of lavender in EO blends containing both, what are suitable for quality control analyses.

Table 4.1. Analytical figures of merit obtained for analytical curves of sweet orange and lavender EOs.

	Sweet orange EO	**Lavender EO**
Equation[a]	$y = (49.69 \pm 1.47)\, x + (1.21 \pm 1.40)$	$y = (-7.03 \pm 0.31)\, x + (-0.10 \pm 0.34)$
R^2	0.9967	0.9948
LOD	2.4 μg mL^{-1}	19.8 μg mL^{-1}
LOQ	8 μg mL^{-1}	65.9 μg mL^{-1}
	0.46 % in blends	3.69 % in blends

[a]Interval calculated considering a confidence level of 95%.

In complement, three different EO blends were prepared and analyzed using the proposed method. Recovery and CV were calculated for sweet orange and lavender EOs in each blend proportion (Table 4.2). Confidence interval was also calculated for each blend proportion recovery value. Recoveries in the range of 71.5 – 128.9% were obtained, what is a satisfactory result, especially considering the complex nature of these blends.

Table 4.2. Recovery (R, %) and coefficient of variation (CV, %) for sweet orange and lavender EOs at three proportions.

Sweet orange: lavender EO proportion (v/v)	**Sweet Orange EO**		**Lavender EO**	
	R (%)[a]	**CV (%)**	**R (%)[a]**	**CV (%)**
25:75	103.9 ± 23.8	9.2	100.2 ± 28.7	11.6
50:50	103.1 ± 7.9	3.1	97.0 ± 10.7	4.4
75:25	94.4 ± 13.7	5.8	92.7 ± 7.7	3.4

[a]Interval calculated considering a confidence level of 95%.

The fluorescence properties of citrus EOs (including sweet orange EO) were previously investigated by Macedo et al. [43], who indicated the non-volatile fraction of the citric EOs, comprising of polymethoxyflavones, coumarins, and furanocoumarins (Figure 4.9), as the fluorescent source. These compounds are present in citrus EO obtained by cold pressing the fruit peels and as previously mentioned, they can serve as markers to quantify citrus EOs in blends of these with EOs from other families. Although the fluorescent characteristics of lavender have also been observed [41], the compounds responsible for it were not found in literature.

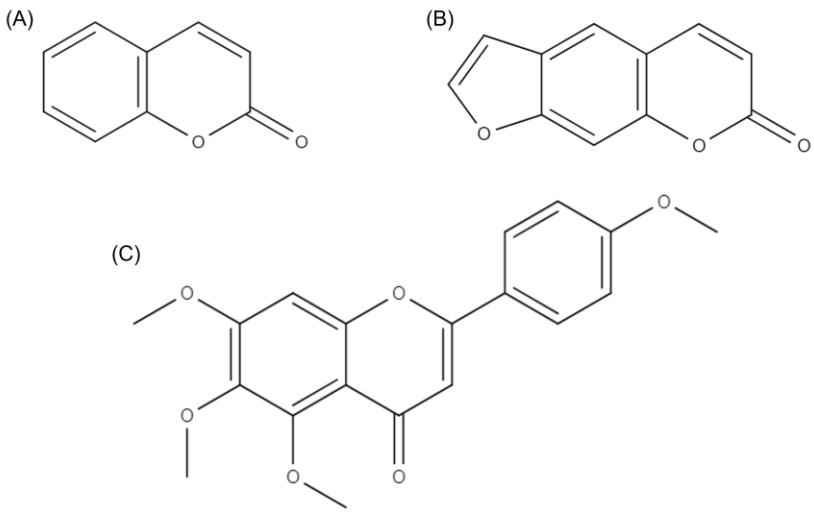

Figure 4.9. Structural formula of (A) coumarin, (B) furocoumarin, and (C) polymethoxiflavones.

4.2
Method application in other blend: preliminary tests

A qualitative test was also conducted with thyme *(Thymus vulgaris)*, an EO from the same family as lavender (Lamiaceae), to preliminarily verify if sweet orange could be quantified in other blends. The thyme and orange blend, although less popular than lavender, also appears in some studies in literature. Some examples include its effect against oxidation in chicken meat [88] and its antibacterial activity against *Salmonella* and *Campylobacter* [89]. Additionally, a blend containing both EOs was able to enhance energy levels in women with fatigue symptoms after Covid-19 recovery [90], what show the potential of this blend in aromatherapy studies.

For the qualitative test with thyme EO, the same procedure applied to the other two EOs was performed. This EO was prepared in a SFME system (50.0 uL

of oily phase 1:4 EO, octan-1-ol, 2.0 mL of water and propan-1-ol until 5.0 mL) and measurements of absorbance and synchronous fluorescence intensity were performed.

Preliminary tests showed that fluorescence intensity of thyme EO prepared in SFME environment was very high compared to sweet orange EO. Because of that, synchronous fluorescence spectral data was obtained for thyme EO at $\Delta\lambda$=100 nm, where sweet orange had its maximum value. Then, optical filter and IFE were corrected, and the first derivative of the corrected spectra was obtained. In Figure 4.10, the corrected and smoothed (Savitzsky Golay, 50 points of window) first derivative spectra for sweet orange and thyme EO is presented.

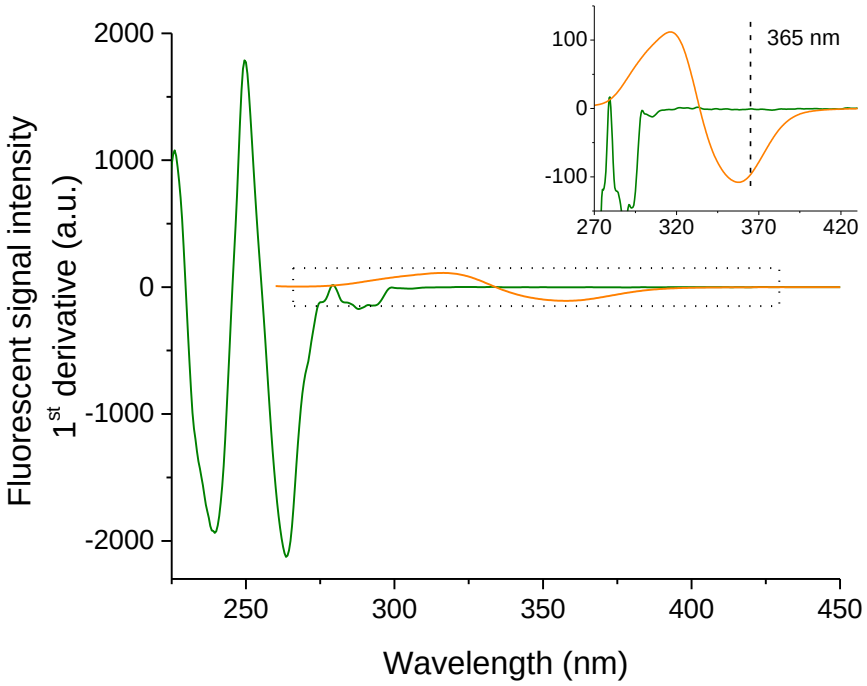

Figure 4.10. Fluorescence signal intensity first derivative at $\Delta\lambda$ = 100 nm for sweet orange and thyme EO samples in SFME systems after IFE correction. SFME was prepared with 50.0 µL of oily phase (containing EO and octan-1-ol, 1:4 v/v), 2.0 mL of water and propan-1-ol up to 5.0 mL final volume for both EOs. Green line represents thyme EO, and orange line represents sweet orange EO. Dotted-line rectangle indicates the area of the graph that was zoomed in and appears in top right corner. Zero cross point for thyme EO is indicated with dashed line.

Although the thyme EO maximum fluorescence intensity is almost 20 times higher than sweet orange EO's, its intensity is close to zero near λ = 365 nm, what creates a favorable region for quantifying sweet orange EO, as shown in the spectrum. A more rigorous study would need to be conducted to identify a suitable region for the quantification of thyme, as it has a more complex and intense

spectrum compared to orange. Despite this, the qualitative test shows potential for the application of the method in quantifying sweet orange in another blend.

4.3
Greenness assessment

The growing concern within the scientific community regarding how environmentally harmful new analytical methods can be has led to the development of greenness assessment metrics based on the relevant GAC parameters. Several green calculators have been used not only to assess how environmentally friendly are the methods developed but also to establish comparison to existing ones in literature. The proposed method was evaluated regarding each one of these metrics, starting with AGREE, which is the most popular and recent, followed by GAPI and lastly, Analytical Eco-Scale.

4.3.1
AGREE calculator

AGREE was the first metric used to analyze the greenness of the proposed method. Although it allows users to assign different weights to each one of the 12 criteria, all of them were considered equal to avoid bias (Figure 4.11) even though some aspects might be considered of greater importance than others depending on the analysis purpose.

Concerning the proposed method for EOs, it stands out primarily in criteria 2, 4, 6, and 8-11, where it achieved maximum scores. This is attributed to the small amount of EO used in sample preparation (10 µL), the few necessary steps (three

or fewer) and absence of derivatization, what is extremely advantageous since, according to GAC, derivatization should be avoided. Besides, the relatively high analytical frequency (120 samples h^{-1}) and the low energy consumption of spectrofluorometer and spectrophotometer also gives the method the higher score in these criteria. In item 10, it was considered that all reagents were bio-based, as 99.0% of the sample is composed of water and propan-1-ol. In criteria 11, it was indicated that no toxic reagents were used, as water and propan-1-ol are not considered overall toxic compared with other reagents also with propan-1-ol/SDS systems as it doesn't possess PBT compounds exceeding 0.1%. Octan-1-ol, although toxic to aquatic environments (which is already accounted for in item 12), also does not contain PBT components exceeding 0.1% according to its SDS. The fact that only 40 µL of octan-1-ol is used for sample preparation make this approximation valid for criteria 11. In criteria 12, besides the toxicity of octan-1-ol to aquatic life, another thing that lowered the score is the fact that propan-1-ol is flammable. However, none of the reagents are considered PBT and none are highly oxidable, explosive or corrosive, what is an advantage considering operator`s safety.

Although the score of 0.79 was considered reasonable, especially given the method's simplicity and the inherent complexity of EO blends, the use of a reduced volume cuvette would decrease the amount of sample required for each analysis. This reduction would minimize waste, contributing to an enhanced overall score. A point of concern that lowers its score is absence of automation where flow injection analysis regime could be used to speed up measurements and automatize but SFME sample preparation could be difficult to produce in-line. However, it can be considered the feasibility of an 'at-line' approach, extracting a partial sample for in

situ quality control. While not as green as the other two, it still outperforms the offline approach. Besides, the degree of automation of the method also lowers the score, as it would be necessary one quartz cuvette for each sample to be analyzed, what would significantly increase the cost required to conduct the analysis.

One thing that is not considered in this metric is the type of generated waste. Even though analytical waste should be as minimal as possible, the type and hazardousness of the residuals as well as their treatment are not taken into account in this metric, being a topic for improvement. Besides that, the generated score can be compared with novel methods in development to correct the flaws and identify trouble spots during experiment planning. The detailed report sheet generated by the AGREE software is presented in the Attachment section, with the individual score for each one of the 12 parameters.

Figure 4.11. AGREE score (0.00 - 1.00) for the proposed method. Each number in the circle represents a different aspect of environmental and process safety: (1) sample treatment, (2) sample amount, (3) device positioning, (4) sample preparation stages, (5) automation and miniaturization, (6) derivatization, (7) waste, (8) analysis throughput, (9) energy consumption, (10) source of reagents, (11) toxicity, and (12) operator's safety.

4.3.2 GAPI

The second metric used in this work was GAPI [7]. For the proposed method, all 15 parameters were carefully evaluated and generated the pictogram seen in Figure 4.12. Three parameters (6,7,8) were left blank since there was no extraction procedure to be applied and no specification was made for this situation in the original article. Besides, 7 out of 12 applicable parameters received the maximum green score (parameters 2-4, 9, 10, 12 and 13), same quantity as AGREE pictogram (parameters 2, 4, 6, 8-11). As the EO blend sample does not need specific storage, preservation, or transport before the analysis, these criteria also received

maximum score. Considering the criteria of NFPA to classify the reagents, both propan-1-ol and octan-1-ol have an NFPA health score of 1, earning a green rating. However, in terms of safety hazards, despite having an NFPA instability score of 0, their flammability scores are 2 and 3, respectively, placing this hazard in the intermediate category. Unlike AGREE, this metric considers if the waste generated received any kind of treatment. In fact, the only parameter that received the red category in the proposed method was this parameter. However, the options fail to consider the hazardous nature of the waste, indicating a potential area for improvement in this metric. This is significant because certain residues pose greater environmental risks than others, which is not the current scenario. Additionally, an advantage of this metric is that, besides possessing more parameters than AGREE, it also considers if the proposed method is adequate for qualitative and quantitative analysis. However, a considerable amount of time is spent constructing this pictogram, which is believed to be a decisive factor for this metric not being as widely used as AGREE. Another point for improvement is that there is no outcome classification system or score generation, so it is only possible to make judgments based on the individual colors of the parameters, since an overall score is not calculated.

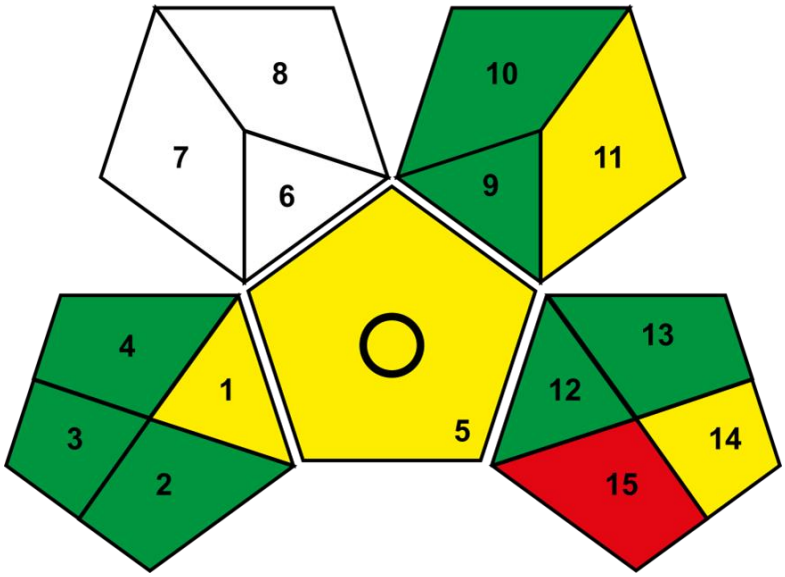

Figure 4.12. GAPI assessment for the proposed method. Each number represents a different aspect of environmental and process safety: (1) sample collection, (2) preservation, (3) transport, (4) storage, (5) direct or indirect type of method, (6) scale of extraction, (7) solvents/reagents used for extraction, (8) additional treatments, (9) amount of reagents and solvents, (10) health hazard, (11) safety hazard, (12) energy, (13) occupational hazard, (14) waste amount and (15) waste treatment. The circle in the middle indicates that procedure is suitable for both qualitative and quantitative analysis.

4.3.3
Analytical Eco-Scale

The Analytical Eco-Scale [6] was also employed to evaluate the method's greenness and verify the compatibility of the results with the first tested metric. Given that the procedure can be conducted in a fume hood, transferred promptly to a volumetric flask, and that the cuvette where sample is placed can be sealed, it was concluded that vapor emission is negligible, and no penalty points were deducted in occupational hazard parameter. A crucial aspect of this method is the absence of

waste treatment, given that the sample, consisting of a dispersion of oily and aqueous phases in SFME, cannot be easily separated. Besides that, the method had only 13 penalty points, scoring a total of 87 points according to this metric, classifying it as excellent in terms of green analysis and being in accordance with AGREE and GAPI previous results. A penalty point table based on the original article [6] regarding the proposed method is presented in Table 4.3. One advantage of this metric compared to the other two, is that it considers all reagents used and deduct points for them individually, what makes it easier to identify points of improvement when designing a method. Besides, the comparison between methods can be done based on the overall score. However, this metric does not possess many parameters to be evaluated, so the method's assessment considering solely Analytical Eco-Scale does not provide as much information as the other two.

Table 4.3. Penalty points considering Analytical Eco-Scale metric [6].

Reagents				
	Number of pictograms	**Signal word**	**Amount of chemical**	**Penalty points**
Ultrapure water	0	0	1	0
Propan-1-ol	3	2	1	6
Octan-1-ol	1	1	1	1
				Σ 7
Instruments				
Energy consumption	< 0.1 kW/h per sample			0
Occupational hazard	Analytical process hermitization			0
Waste	1-10 mL			3
	No treatment			3
				Σ 6
Total penalty points: 13				
Analytical Eco-Scale score: **87**				

Overall, all three metrics suggest that the proposed method adopts an environmentally friendly approach despite employing different classification systems. Many other metrics are present in literature for the greenness assessment, however, these three were considered the most suitable considering the method's nature. The use of three metrics to assess the method's eco-friendliness and their agreement among each other reinforce its greens aspect.

5
Conclusion

The quality control of complex samples like EO blends remains a challenging task that requires attention from the scientific community. In this work, a straightforward and cost-effective method aligned with GAC was developed for quantification of sweet orange and lavender EOs contents in blends containing both. The use of SFMEs as sample preparation allowed for a low consumption of EOs, which is economically significant. Additionally, there was an increase in the fluorescence when compared to the EO alone. Regarding analytical parameters of merit, the LOD and LOQ obtained were 2.4 μg mL^{-1} and 8.0 μg mL^{-1} (sweet orange EO) and 19.8 μg mL^{-1} and 65.9 μg mL^{-1} (lavender EO). It was possible to quantify up to 0.5% and 3.7% of sweet orange and lavender, respectively, in blends containing both EOs. The recovery values in the 25:75, 50:50 and 75:25 proportions were 103.9 ± 23.8 (CV = 9.2 %), 103.1 ± 7.9 (CV = 3.1 %) and 94.4 ± 13.7 (CV = 5.8 %) respectively, for sweet orange and 100.2 ± 28.7 (CV =11.6%), 97.0 ± 10.7 (CV = 4.4 %) and 92.7 ± 7.7 (CV = 3.4 %), respectively, for lavender EOs.

Greenness assessments were performed using Analytical Eco-Scale, GAPI and AGREE. The first one generated a score of 87 out of 100, and in the second and third metrics, seven out of twelve utilized parameters achieved the highest score, with an AGREE total score of 0.79 out of 1.00. The resulting score for the three metrics along with the acceptable results for the analytical parameters of merit position this approach as both analytically satisfactory and aligned with the principles of GAC.

Although the method succeeded in its application for sweet orange and lavender EO blend and showed potential in the case of sweet orange and thyme, some considerations for its use must be addressed. Firstly, the method is particularly useful for analyzing blends containing EOs from different families. However, in blends containing species from the same family, the similarity in their compositions and fluorescent characteristics can make it challenging to find regions where the signal is solely attributable to one component. Additionally, it should be considered that synchronous fluorescence with the application of the first derivative may not always resolve band overlap, especially in cases of significant intensity differences. Therefore, preliminary studies of the specific system should be conducted to assess the feasibility of its application.

Besides these considerations, the developed method has the potential to be used as a quality control procedure for blends of sweet orange and lavender EOs. Since there is no standard procedure for this analysis, the usage of a spectrofluorometer and a spectrophotometer in the quality control laboratory is a feasible approach for real-world applications without the need for separation techniques resulting in a reduction in costs and in waste generation.

6

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7
Attachments

A
Participation in events

1) **47ª Reunião Anual da Sociedade Brasileira de Química (May/24)**

   Co-authorship of work presented in banner.

2) **II Seminário da Pós-Graduação do CTC/PUC-Rio (Apr/24)**

   Video and banner presentation.

3) **Programme for Youth on Peaceful uses of chemistry – OPCW (Apr/24)**

   Participated as an observer in the training.

4) **VII Jornada de Pós-Graduação em Química - PUC/Rio (Nov/23)**

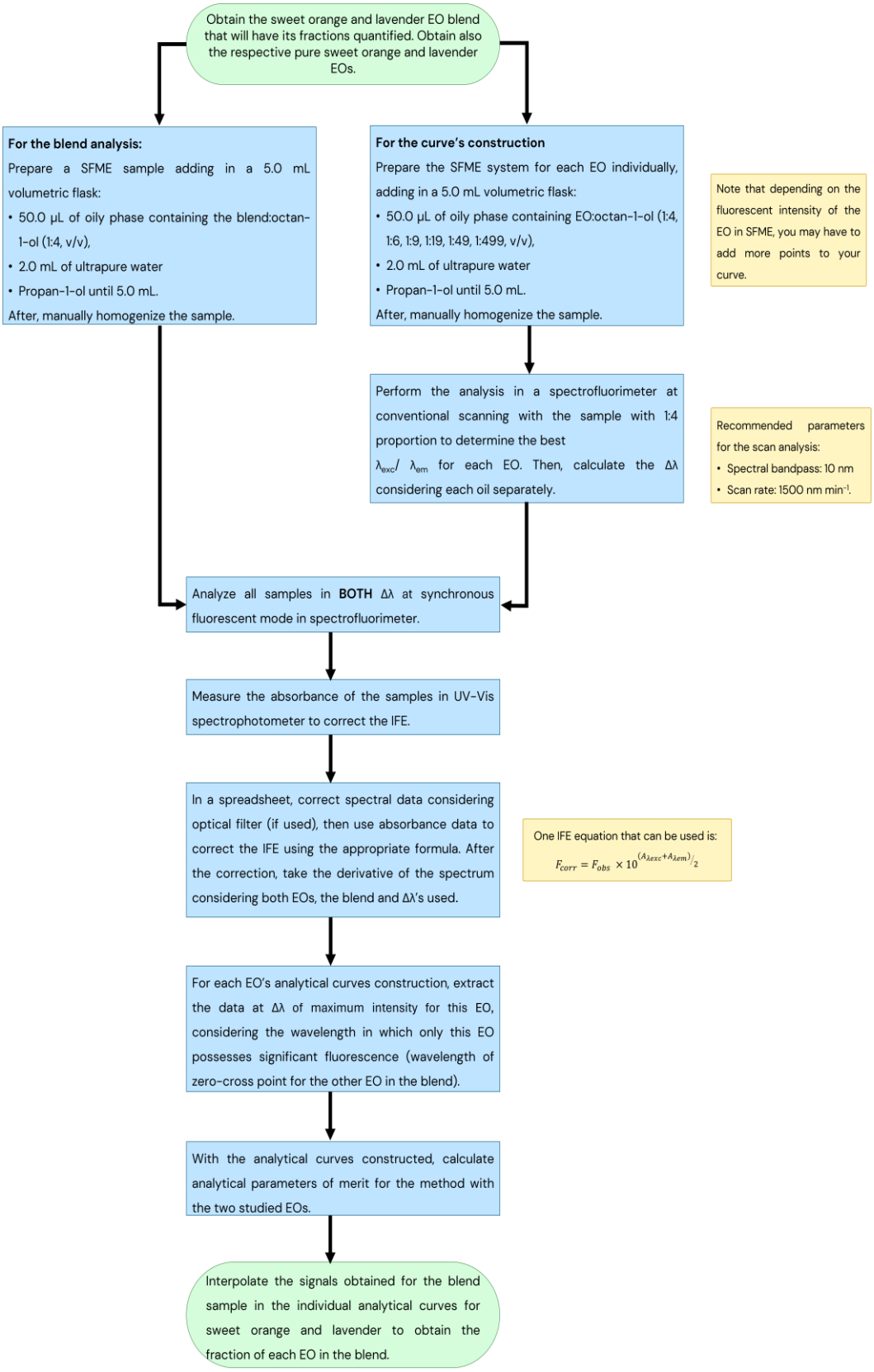
   Oral presentation.

5) **11º Simpósio Brasileiro de Óleos Essenciais (Nov/23)**

   Oral presentation. Rewarded with best oral presentation of the event.

B

General procedure for quantification of EO in blends



C

AGREE report sheet

Analytical Greenness report sheet

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Criteria	Score	Weight
1. Direct analytical techniques should be applied to avoid sample treatment.	0.6	2
2. Minimal sample size and minimal number of samples are goals.	1.0	2
3. If possible, measurements should be performed in situ.	0.33	2
4. Integration of analytical processes and operations saves energy and reduces the use of reagents.	1.0	2
5. Automated and miniaturized methods should be selected.	0.5	2
6. Derivatization should be avoided.	1.0	2
7. Generation of a large volume of analytical waste should be avoided, and proper management of analytical waste should be provided.	0.48	2
8. Multi-analyte or multi-parameter methods are preferred versus methods using one analyte at a time.	1.0	2
9. The use of energy should be minimized.	1.0	2
10. Reagents obtained from renewable sources should be preferred.	1.0	2
11. Toxic reagents should be eliminated or replaced.	1.0	2
12. Operator's safety should be increased.	0.6	2

D

Published paper in the scope of this dissertation

Title: Green method for quantification of lavender and sweet orange essential oils in blends by synchronous fluorescence first derivative.

Authors: Beatriz Serrão Monteiro Bastos, Rosana Candida Macedo, Ricardo Queiroz Aucelio.

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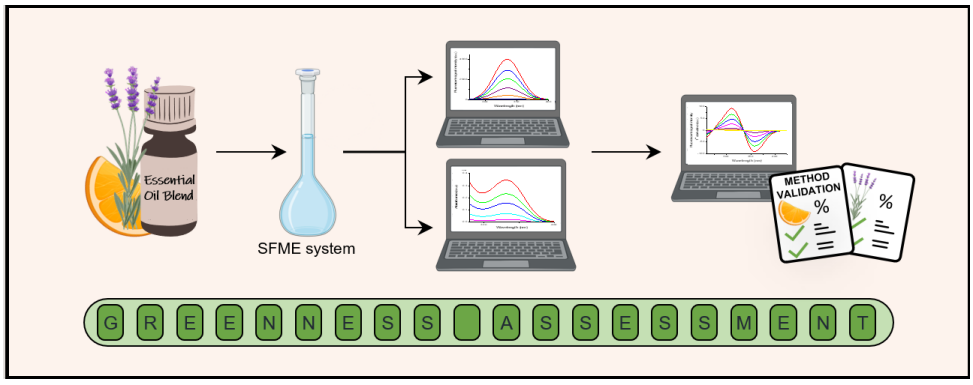
Graphical Abstract:



The full article is presented in the following pages.

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Green method for quantification of lavender and sweet orange essential oils in blends by synchronous fluorescence first derivative

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ABSTRACT

A simple green method for the quantification of sweet orange and lavender essential oils (EOs) content in blends was developed using synchronous fluorescence of the first derivative spectra and sample preparation by surfactant-free microemulsions (SFMEs). Excitation and emission wavelengths pairs ($\lambda_{ex}/\lambda_{em}$) were determined for both EOs (at 336/436 nm for sweet orange and at 330/388 nm for lavender). Optimization was conducted to establish the condition to prepare the SFMEs systems: 50 μL of oily phase containing EO diluted in octan-1-ol (1:4, v/v), 2.0 mL of water and propan-1-ol up to 5.0 mL final volume. Different proportions of EO in the oily phase were evaluated for analytical curves construction. Synchronous fluorescence measurements were performed and the acquired data were corrected by inner filter effect. Considering the first derivative of the synchronous scanning, data were extracted at 316.8 nm ($\Delta\lambda$ = 100 nm) and 352.0 nm ($\Delta\lambda$ = 58 nm) for sweet orange and lavender EOs, respectively, with correlation coefficients above 0.99. Limits of quantification of 7.97 μg mL^{-1} and 65.80 μg mL^{-1} were obtained indicating that 0.46% of sweet orange and 3.69% of lavender EOs can be quantified in mixtures. Simulated blends (25:75%, 50:50% and 75:25%, volume proportions) were also evaluated with recoveries of 71.5 – 128.9% and coefficient of variation between 3.1% – 11.6%. Additionally, the method's greenness was evaluated through three green metrics (Analytical Eco-Scale, GAPI, and AGREE) with consistent results.

1. Introduction

Aromatherapy has been used as an alternative approach for the treatment of various conditions and many of the reported procedures are based on essential oils (EOs). Although the term "aromatherapy" was introduced in the 1930's [1], historical evidence suggests that such a complementary therapy has found practice for at least 6000 years in India, China, and Egypt [2]. Currently, aromatherapy is used to improve one's health in physical, mental, and emotional aspects with EOs administered by inhalation, massage, or simple skin application [3]. Studies have shown its effectiveness in reducing perceived stress and depression, improving the quality of sleep and memory, and even controlling pain and chronic diseases [2–5].

Orange EO is one of the most commercialized EOs in the world as it is obtained by cold pressing of fruit peels, an important by-product of the juice industry [6,7]. It has a higher production rate compared to other oils, what makes its commercial value more accessible to consumers. More specifically, sweet orange EO (*Citrus sinensis* or *Citrus aurantium dulcis*) [8] is known for its antioxidant, cytotoxic, bactericidal, and antimicrobial activities [9,10]. In aromatherapy, this oil has great value due to its anxiolytic effect [11–13] and can be used alone or in combination with other EO varieties, such as lavender (*Lavandula angustifolia)* [8] that is also known for its effect against anxiety disorders. Studies are found in the literature not only comparing the effects of these two oils separately [13–16], but also in combined formulations [17–21].

A study was conducted where patients were exposed to orange and lavender EOs and a mixture of the two showed enhancement in self-orientation associated with cognitive function [17]. Another work reported that a one-month period utilization of a blend of sweet orange and lavender EOs (in a 1:1 proportion) not only enhanced sleep quality of dialysis patients but also reduced fatigue [18]. Other more complex mixtures containing sweet orange and lavender in the composition also have been studied with satisfactory outcomes. [19–21]

Currently, it is common to find fragrance houses selling mixtures of EOs, commercially known as blends. However, these products are usually sold without specifying the fraction of each EO present in their

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composition. In the case of blends involving the mixture of sweet orange and lavender EOs it's important to consider the distinct costs associated with each of these EOs. In this sense, counterfeit formulations may present smaller proportions of the higher value EO than what is reported thus harming consumers. The present work intends to develop a simple and rapid method for the quantification of sweet orange EO and lavender EO through synchronous spectrofluorimetry using the first derivative spectra to enhance selectivity as a limited number of works with this purpose is found in literature.

Due to the complex composition [22], quantification of EOs in blends involve separation techniques, coupled with complex mathematical data treatment [23,24], what may limit their use in routine analysis. De Godoy, et al. [23] used two-dimensional gas chromatography with flame ionization detection (GC×GC-FID) coupled with multivariate quantitative models (multivariate curve resolution and alternating least squares, MCR-ALS) for quantitative analysis of EOs in perfume. Lebanov, et al. [24] also quantified EOs in blends using MCR-ALS algorithms. In this case, data sets were acquired on average mass spectrum obtained from gas chromatography with mass spectrometry (GC–MS). Although both methods have demonstrated success in quantifying EOs in complex mixtures, they require costly equipment maintenance and operators with a high level of expertise. Additionally, the complexity of the mathematical models used demands personnel proficient in chemometrics, which is not always feasible for routine analysis. Therefore, it is necessary to develop new methods tailored for cost-effective routine analysis, employing simple analytical techniques, and utilizing simplified mathematical treatments.

In this context, spectrofluorimetry is as an alternative technique [25] that attend the mentioned requisites to enable simple routine quality control of EOs and blend of EOs as they may present specific fluorophores in their compositions. Feudjio, et al. [26,27] employed 3D fluorescence spectroscopy analysis coupled with chemometric treatment to distinguish high-value EOs adulterated through blending with more affordable market-value oils. In the most recent work [27], these same authors evaluated the degree of adulteration in neroli EO with sunflower vegetable oil, employing a multilayer perceptron (MLP) network. However, no quantitative analysis was conducted for the mixtures of EOs.

For the present work, a simple analytical method using synchronous fluorescence spectroscopy is proposed, with a focus on quantifying blends of sweet orange and lavender EOs. The method explores specific fluorescence characteristics of each oil. In this case, synchronous fluorescence using the first derivative of the emission spectra was employed to minimize spectral overlap, greatly increasing selectivity [28,29]. However, in this context, the oily nature of the samples must be considered as their high viscosity and opacity hinder direct analysis of oils, which are essentially the reasons why fluorescence spectroscopy is not commonly used for this purpose. In order to circumvent this, oils were prepared as surfactant-free microemulsions (SFMEs) systems as they are transparent and thermodynamically stable, making two or more immiscible liquids (in this specific case, aqueous and oily phases) stabilized by the addition of short-chain alcohols such as methanol, ethanol, or propan-1-ol, which are used as amphiphilic solvents [30]. Additionally, the formation of micelle-like aggregates restricts fluorophore molecular vibration also protecting them from dynamic quenching, thus decreasing non-radiative processes, consequently enhancing the measured fluorescence signal [25]. Studies conducted by Macedo, et al. [31] have shown that pseudo-ternary systems with EO and octan-1-ol in the oily phase, together with propan-1-ol and water can substantially increase the fluorescence intensity, lowering sample amount required for analysis. In addition, the proposed method is in line with most of the green chemistry principles, for instance by minimizing both waste and the use of hazardous reagents. The low sample consumption is a significant factor, considering the high value of these products. Additionally, a critical discussion about the method's greenness is accessed in this work through green calculators available in the literature [32–34].

2. Experimental

2.1. Reagents and chemicals

Sweet orange (*Citrus aurantium dulcis*) and lavender (*Lavandula angustifolia*) essential oils, both commercially available, were obtained from the same Brazilian supplier. For SFME systems preparation, ultrapure water (Millipore, USA), propan-1-ol (Merck, Germany) and octan-1-ol (Sigma-Aldrich, USA) were used.

2.2. Instruments and apparatuses

Milli-Q gradient A10 ultra-purifier (Millipore, USA) was the source of ultrapure water. Fluorescence measurements were made using a Perkin-Elmer (UK) LS 55 luminescence spectrophotometer. Absorbance measurements were made on a Varian (USA) Cary 100 spectrophotometer. Fluorescence and absorbance spectra were respectively obtained through a FL WinLab software, version 4.00.02 and a Cary WinUV software, version 3.00(182). Optical reflective density filters (Newport, USA) were used for fluorescence attenuation. Data treatment and figures were done using OriginLab software (OriginLab Corporation, version 2023b, USA).

2.3. Procedures

2.3.1. SFME preparation

The SFME preparation procedure was adapted from Macedo et al. [35]. Each system consisted of 50 μL of an oily phase (EO diluted in octan-1-ol, 1:4 v/v), 2.0 mL of ultrapure water, and propan-1-ol until a final volume of 5.0 mL. The reagents were added in the mentioned order in a volumetric flask, followed by manual agitation and spontaneous formation of the SFME. This condition was achieved after carefull optimization. For method application, SFMEs were prepared with simulated blends of sweet orange and lavender EOs at three percent proportions (25:75, 50:50, 75:25 v/v). All experiments were performed in triplicate.

2.3.2. Univariate optimization

The univariate optimization was conducted using sweet orange EO since this oil presented higher fluorescence. Preliminary tests were performed from the initial condition: 60 μL of oily phase (EO: octan-1-ol, 1:2 v/v), 2.0 mL of water and adjusting final volume with propan-1-ol until 5.0 mL [35]. For univariate optimization, a Stokes shift ($\Delta\lambda$) of 100 nm was used for obtaining synchronous fluorescence signal. Different volumes of oily phase (10, 20, 30, 40, 50, and 60 μL), and different proportions of EO in the oily phase (1:0, 1:1, 1:2, 1:3, 1:4, 1:7 and 1:10 v/v in octan-1-ol) were considered. The condition with the highest fluorescence signal aligned with linear response range was set for water amount optimization (0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 3.5 mL).

2.3.3. Spectroscopic analysis

Fluorescence spectra were acquired with a 10 nm spectral bandpass for both excitation and emission, and 1500 nm min^{-1} scan rate. Excitation and emission fluorescence spectral ranges were respectively from 250 to 400 nm and from 350 to 550 nm. For synchronous fluorescence spectroscopy analysis, measurements were made from 200 to 500 nm, using $\Delta\lambda$s of 58 and 100 nm. An optical reflective density filter of 10% transmittance (nominal value) was used to attenuate fluorescence intensity. All measurements were made with the same optical filter. To correct the inner-filter effect [36], absorbance spectra (from 200 to 600 nm) were acquired with scan rate of 300 nm min^{-1} and data interval of 0.5 nm.

2.3.4. Data treatment

The fluorescence intensity was adjusted based on the corresponding optical filter calibration values. Then, inner-filter effect correction [36] was made by applying the general formula explicit in Eq. (1), where F_{corr} corresponds to fluorescent signal intensity after inner-filter correction, while F_{obs} corresponds to raw data only with optical filter correction. $A_{\lambda exc}$ and $A_{\lambda em}$ corresponds to absorbance measurements in the excitation and emission wavelength, respectively.

$$F_{corr} = F_{obs} \times 10^{(A_{\lambda exc} + A_{\lambda em})2} \quad (1)$$

The first derivatives of the corrected spectra were calculated and smoothed with Savitzky-Golay algorithm with a window size of 100 data points.

2.3.5. Method validation

For method validation, samples were prepared using sweet orange and lavender EOs, separately, at different volume proportions with octan-1-ol. Conditions named from (A-F) with ratios of 1:4 (A), 1:6 (B), 1:9 (C), 1:19 (D), 1:49 (E) and 1:499 (F) v/v were used. These conditions correspond to concentrations of 1.72 (A), 1.23 (B), 0.86 (C), 0.43 (D), 0.17 (E) and about 0.02 (F) mg mL^{-1} for sweet orange EO and 1.78 (A), 1.27 (B), 0.89 (C), 0.45 (D), 0.18 (E) and about 0.02 (F) mg mL^{-1} for lavender EO, as summarized in Table A1. Synchronous fluorescence spectroscopy analyses were performed for each sample. First derivative spectra of the synchronous spectra (after treatment) were obtained. To construct the analytical curves, data were collected at zero cross points for each oil (316.8 nm for sweet orange EO and 352.0 nm for lavender EO) that are the wavelengths that spectral contribution of one oil is minimum when measuring fluorescence from the other oil.

Analytical figures of merit were calculated including limit of detection (LOD) (Eq. (2)) and limit of quantification (LOQ) (Eq. (3)) where s_b corresponds to blank standard deviation ($n = 7$) and m corresponds to the analytical curve sensitivity. Linear response was evaluated through the coefficient of determination (R^2) with residual constructed for homoscedasticity determination. Simulated blends were used to assess the effectiveness of the proposed method.

$$LOD = 3 \times s_b/m \quad (2)$$

$$LOQ = 10 \times s_b/m \quad (3)$$

2.3.6. Greenness evaluation

Method's greenness was evaluated through different metrics available in the literature, including Analytical Eco-Scale [32], Green Analytical Procedure Index (GAPI) [33] and Analytical GREEnness Metric Approach (AGREE) [34]. A critical discussion about the method's application in these green calculators was accessed as well as advantages and disadvantages regarding each one.

3. Results and discussion

3.1. Univariate optimization

Pseudo-ternary systems involving the oily phase (EOs in octan-1-ol), water and propan-1-ol have proven to be suitable for dispersing and stabilizing EO in order to conduct spectrofluorometric analysis. These systems (in this case Surfactant-Free Microemulsions or SFME) require lower amounts of EO, which is advantageous considering the high aggregated value of such samples. Besides, microemulsions are microenvironments where less polar fluorophores allocate, providing large fluorescence enhancement factors [31,35].

Preliminary studies were conducted to establish the best characteristic wavelength pairs ($\lambda_{exc}/\lambda_{em}$) for each type of EO in SFMEs. Experimental condition, previously described by Macedo et al. [35], was used as a starting point. In this case, SFME was prepared with 60 µl of oily phase (EO:octan-1-ol, 1:2 v/v) 2.0 mL of water and propan-1-ol until final volume of 5.0 mL. Under these conditions, the maximum excitation (λ_{ex}) / emission (λ_{em}) wavelength pairs were 336/436 nm for sweet orange EO and 330/388 nm for lavender EO, showing a clear spectral difference between these two EOs. As a result, the Stokes shift ($\Delta\lambda = \lambda_{em} - \lambda_{ex}$) were 58 nm and 100 nm respectively (Fig. 1).

After determining the characteristic $\Delta\lambda$ value, optimization was conducted using sweet orange EO as it presented the higher fluorescence response. First, the oily phase volume and dilution factor of EO in SFME were evaluated, with water amount fixed at 2.0 mL. Volumes of 10, 20, 30, 40, 50 and 60 µL of the oily phase were tested. For each condition, different volume proportions of EO and octan-1-ol in the oily phase were used: 1:0 (100% EO), 1:1 (50% EO), 1:2 (33.3% EO), 1:3 (25% EO) 1:4 (20% EO), 1:7 (12.5% EO) and 1:10 (9.1% EO). These samples were analyzed by fluorescence spectroscopy to establish robust conditions for SFME formation, taking into consideration the response in terms of signal intensity. Synchronous fluorescence spectra intensities were measured at $\Delta\lambda = 100$ nm and the intensities recorded in function of volume of the oily phase and percentage of EO in the oily phase can be seen in Fig. 2A.

From Fig. 2A, it becomes evident that an increase in EO within a constant volume of oily phase leads to a rise in fluorescence intensity. However, beyond 25% of EO (equivalent to a 1:3 proportion), the linear response of the systems begins to decrease, particularly noticeable in larger volumes of the oily phase. Given the objective of maximizing fluorescence intensity to enhance method's sensitivity while reducing volume of oily phase, due to the high intrinsic value of the EOs sample, the 50 µL oily phase volume was chosen. This decision was based on the observation that the linear response of the last four data points surpassed that of 60 µL, and the intensity was higher than that achieved with 40 µL (Fig. 2B).

Additionally, the 1:4 proportion of EO:octan-1-ol was chosen as a compromise condition to ensure it was within a linear working range. Evaluating the water content under this condition (50 µL of oily phase, EO:octan-1-ol, 1:4 v/v), turbidity was observed in the system upon adding a volume of 3.5 mL of water (Fig. A.1). To ensure that the system would be within a robust range for the formation of SFME, it was chosen to maintain a volume of 2.0 mL of water. In summary, compromise condition used for the subsequent experiments was set at 2.0 mL of water and 50 µL of oily phase (EO:octan-1-ol, 1:4 v/v).

3.2. Spectrofluorimetric analysis and data treatment

With a compromise condition set, analytical curves were constructed regarding sweet orange and lavender EOs. Synchronous fluorescence spectral data were first organized in spreadsheets for optical filter correction, then absorbance data was used for inner-filter effect correction (Eq. 1). The obtained smoothed spectra for sweet orange ($\Delta\lambda = 100$ nm) and lavender ($\Delta\lambda = 58$ nm) EOs are present in Fig 3A-B, respectively. The first derivative of the corrected spectra in the two cases were also obtained (Fig 3C-D) and smoothed (Savitzky Golay, 100 data points) for the analytical curve construction.

To select the optimal wavelength for data extraction, the concept of "zero cross point" was considered, where the total intensity at a certain wavelength is determined by only one component of the binary mixture, as the other has a negligible value regardless of concentration [25]. This occurs because the derivative of the maximum fluorescence intensity intersects the x-axis, and the total intensity is solely attributable to the other component. Using this concept, at $\Delta\lambda = 100$ nm, the wavelength of 316.8 nm was considered the zero-cross point for lavender and used to construct the sweet orange curve. Similarly, at $\Delta\lambda = 58$ nm, the selected wavelength was 352 nm for lavender curve construction as highlighted in Fig 3C-D. The use of this mathematical is adequate for discriminating signals associated with different compounds when analyzing first derivative spectra [25]..

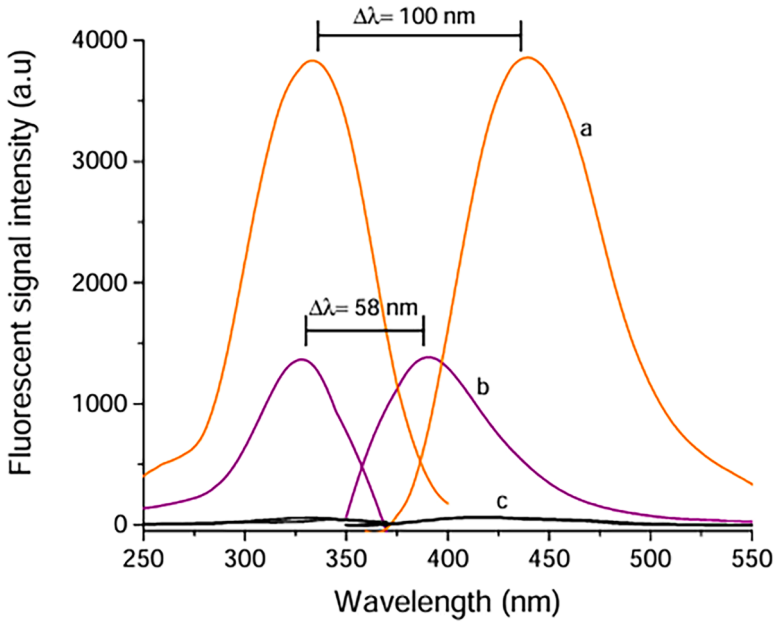

Fig. 1. Fluorescence excitation and emission spectra obtained for sweet orange and lavender EOs in SFME media. SFME consisting of 60 μL of oily phase (EO : octan-1-ol, 1:2 v/v), 2.0 mL of ultrapure water and final volume adjusted with propan-1-ol until 5.0 mL. The spectra represent (a) sweet orange essential oil, (b) lavender essential oil and (c) blank measurements.

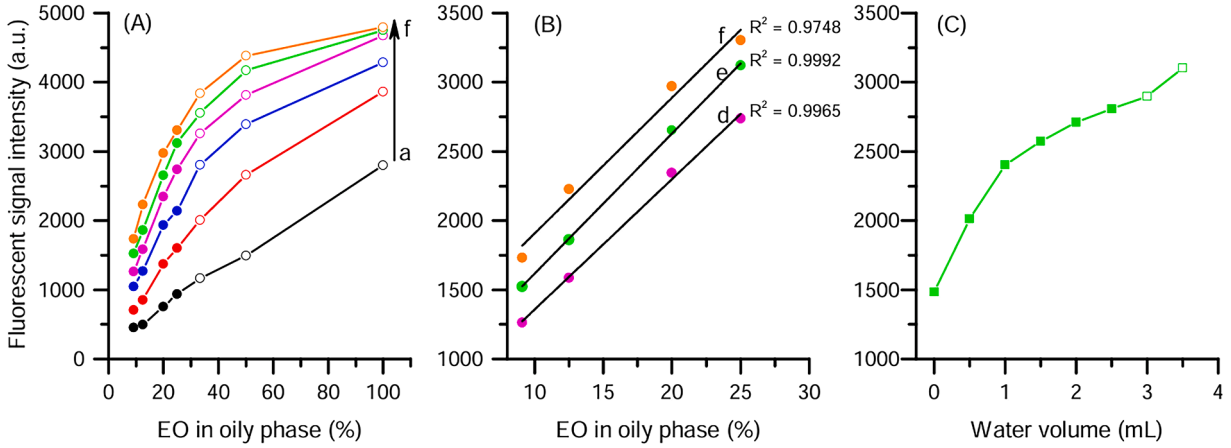

Fig. 2. Optimization experiments for establishing the formation conditions of SFME systems from sweet orange essential oil: (A) Fluorescent signal intensity obtained for systems with six volumes of oily phase (a) 10, (b) 20, (c) 30, (d) 40, (e) 50 and (f) 60 μL using different dilutions of EO in oily phase (1:0 or 100% EO; 1:1 or 50% EO; 1:2 or 33.3% EO, 1:3 or 25% EO; 1:4 or 20% EO; 1:7 or 12.5% EO and 1:10 or 9.1% EO). (B) Zoom at the linear range obtained for (d-f) with the coefficient of determination (R^2) highlighted. 2.0 mL of ultrapure water and propan-1-ol were used for SFME systems formation. (C) Evaluation of water amount effect (0.0 to 3.5 mL) for the condition with 50 μL of oily phase and 20% of EO in oily phase (1:4 proportion). Unfilled markers indicate systems with non-satisfactory visual aspect or linearity. Measurements were made in synchronous scan mode (200 to 500 nm, $\Delta\lambda = 100$ nm).

3.3. Method validation

In order to confirm the effectiveness of the inner-filter effect correction upon curve linear response, the sweet orange EO analytical curve was constructed with the same parameters without the use of correction, obtaining the determination coefficient (R^2) of 0.9690. Analytical curve and the associated residual plots are presented in Fig A.2**(A-C)**. In this case, it is observed that there is an absence of random distribution of residuals around the y-axis. Besides, Fig A.2**(C)** also indicates that most of the residuals are not concentrated around the zero point, as expected, showing the importance of inner-filter effect correction before constructing the analytical curves.

Considering the inner-filter effect correction, the same procedure was performed not only for sweet orange EO but also for lavender EO. Upon ordinary least square linear regression, a correlation coefficient (R^2) of 0.9967 and 0.9948 were obtained for sweet orange EO and lavender EO analytical curves, respectively. A histogram of the residual plot for each group data was also obtained, and in both cases, a random distribution of points around zero was obtained (Fig. 4A-B). The R^2 increases substantially and the residual plots for sweet orange EO analytical curve indicates a more adjusted and selective response when compared to Fig A.2**.**

Moreover, the analytical figures of merit were obtained for the group data corrected for inner-filter effect, including LOD and LOQ (Table 1). The LOQ shows the possibility of quantifying a minimum of 0.46% of sweet orange and 3.7% of lavender in essential oil blends containing both, what are suitable for quality control analyses.

In complement, three different EO blends were prepared and analyzed using the proposed method. Recovery and coefficient of variation (CV) were calculated for sweet orange and lavender EOs in each

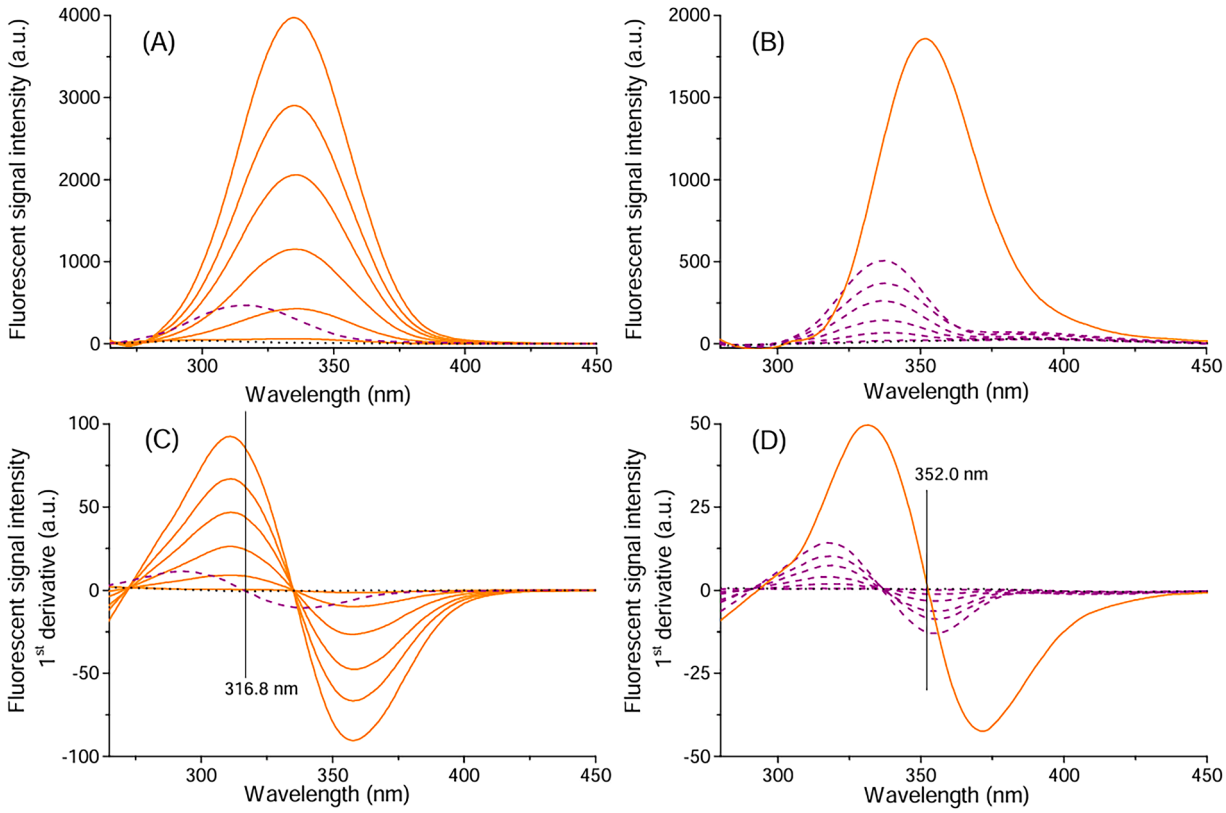

Fig. 3. Synchronous fluorescent signal intensity at (A) $\Delta\lambda = 100$ nm and (B) $\Delta\lambda = 58$ m for sweet orange and lavender EO samples in SFME systems after inner-filter effect correction. Fluorescence intensity first derivative at (C) $\Delta\lambda = 100$ nm and (D) $\Delta\lambda = 58$ m respectively for sweet orange and lavender EO samples in SFME systems after inner-filter effect correction. At $\Delta\lambda = 100$ nm, proportions of sweet orange EO: octan-1-ol of 1:4, 1:6, 1:9, 1:19, 1:49, 1:499, v/v and proportion of lavender EO: octanol, 1:4, v/v were used. At $\Delta\lambda = 58$ nm, proportion of sweet orange: octanol, 1:4, v/v and proportions of lavender EO: octan-1-ol of 1:4, 1:6, 1:9, 1:19, 1:49, 1:499, v/v were used. Zero cross point is highlighted in the spectra for (C) lavender EO and (D) sweet orange. SFME: 50 μL of oily phase (containing EO and octan-1-ol), 2.0 mL of water and propan-1-ol up to 5.0 mL final volume. Solid line represents sweet orange essential oil measurements, dashed line represents lavender essential oil measurements and dotted line represents blank.

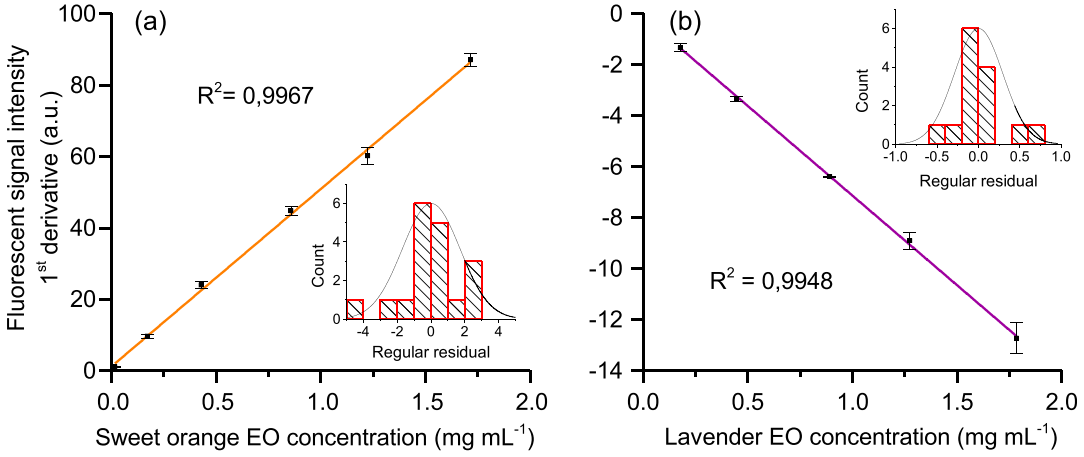

Fig. 4. Analytical curves (ordinary least square linear regression) based on results obtained for (a) sweet orange and (b) lavender EOs analysis. For analytical curve construction, the fluorescence intensities were collected at $\lambda = 316.8$ and 352.0 nm from the first derivative of the synchronous scanning spectrum ($\Delta\lambda = 100$ and 58 nm for sweet orange and lavender EOs, respectively). Concentration varying from (A) to (F) and from (A) to (E) were used to construct sweet orange and lavender EO analytical curves, respectively. Residual plots are presented for each graph.

blend proportion (Table 2). Confidence interval was also calculated for each blend proportion recovery value. Recoveries in the range of 71.5 – 128.9% were obtained, what is a satisfactory result, especially considering the complex nature of the blends.

The fluorescent properties of citrus oils (including sweet orange EO) were previously investigated by Macedo et al. [35], who indicated the non-volatile fraction of the citric EOs, comprising of polymethoxyflavones, coumarins, and furanocoumarins, as the source. These compounds are characteristic of all citrus oils obtained by cold pressing the fruit peels. The components responsible for fluorescent properties in lavender EO were not reported in literature.

The developed method has the potential to be used as a quality

Table 1
Analytical figures of merit obtained for analytical curves of sweet orange and lavender EOs.

	Sweet orange EO	Lavender EO
Equation[a]	$y = (49.69 \pm 1.47)\,x + (1.21 \pm 1.40)$	$y = (-7.03 \pm 0.31)\,x + (-0.10 \pm 0.34)$
R^2	0.9967	0.9948
LOD	2.39 μg mL^{-1}	19.8 μg mL^{-1}
LOQ	7.97 μg mL^{-1}	65.9 μg mL^{-1}
	0.46% in blends	3.69% in blends

[a] Interval calculated considering a confidence level of 95%.

Table 2
Recovery (R,%) and coefficient of variation (CV,%) for sweet orange and lavender EOs at three proportions.

Sweet orange: lavender EO proportion (v/v)	Sweet Orange EO		Lavender EO	
	R (%)[a]	CV (%)	R (%)[a]	CV (%)
25:75	103.9 ± 23.8	9.22	100.2 ± 28.7	11.56
50:50	103.1 ± 7.9	3.10	97.0 ± 10.7	4.44
75:25	94.4 ± 13.7	5.78	92.7 ± 7.7	3.39

[a] Interval calculated considering a confidence level of 95%.

control procedure for blends of sweet orange and lavender essential oils. Since there is no standard procedure for this analysis, the usage of a spectrofluorometer and a spectrophotometer in the quality control laboratory is a feasible approach for real-world applications without the need for separation techniques. With this, there would be a diminish of costs and reduction in waste generation.

3.4. Greenness evaluation

The growing concern within the scientific community regarding how environmentally harmful new analytical methods can be has led to the development of greenness assessment metrics based on relevant green chemistry parameters. Some to be mentioned include energy consumption, toxicity and hazardousness of reagents, number of steps in analysis procedure, need for derivatization and waste generation. Several green calculators have been used not only to assess how environmentally friendly are the methods developed but also to establish comparison to existing ones in literature. Some green calculators to be mentioned include Analytical Eco-Scale, GAPI and, more recently, AGREE.

Although AGREE was only published in 2020, it has shown the highest citation growth rate in recent years [37], what may be attributed not only to its ease of access and use but also to its straightforward comprehensibility. This calculator was created as an attempt to overcome gaps and disadvantages regarding previously created metrics, providing more sensitive and less general information, taking into consideration the 12 principles of green analytical chemistry [34]. AGREE produces a user-friendly pictogram of easy comprehension inside and outside scientific community. Although this metric allows users to assign different weights to each one of the 12 criteria, all of them were considered equal to avoid bias (Fig 5A) even though some aspects might be considered of greater importance than others depending on the analysis purpose.

Concerning the proposed method for EOs, it stands out primarily in criteria 2, 4, 6, and 8–11, where it achieved maximum scores. This is attributed to the small amount of oil used in sample preparation (10 μL), the few necessary steps (three or fewer), absence of derivatization, and the relatively high analytical frequency (120 samples h^{-1}). In item 10, it was considered that all reagents were bio-based, as 99.0% of the sample is composed of water and propan-1-ol. In criteria 11, it was indicated that no toxic reagents were used, as water and propan-1-ol are not considered acutely toxic. Octan-1-ol, although toxic to aquatic

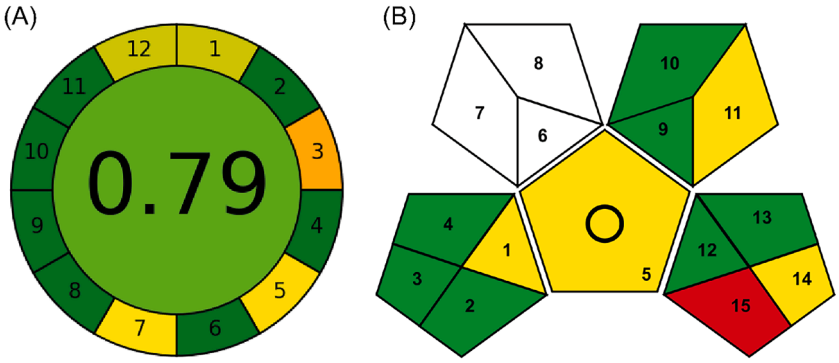

Fig. 5. (A) AGREE score (0.00 - 1.00) for the proposed method. Each number in the circle represents a different aspect of environmental and process safety: (1) sample treatment, (2) sample amount, (3) device positioning, (4) sample preparation stages, (5) automation and miniaturization, (6) derivatization, (7) waste, (8) analysis throughput, (9) energy consumption, (10) source of reagents, (11) toxicity, and (12) operator's safety. (B) GAPI assessment for the proposed method. Each number represents a different aspect of environmental and process safety: (1) sample collection, (2) preservation, (3) transport, (4) storage, (5) direct or indirect type of method, (6) scale of extraction, (7) solvents/reagents used for extraction, (8) additional treatments, (9) amount of reagents and solvents, (10) health hazard, (11) safety hazard, (12) energy, (13) occupational hazard, (14) waste amount and (15) waste treatment. The circle in the middle indicates that procedure is suitable for both qualitative and quantitative analysis.

Table 3
Penalty points considering Analytical Eco-Scale metric [32].

Reagents				
	Number of pictograms	Signal word	Amount of chemical	Penalty points
Ultrapure Water	0	0	1	0
Propan-1-ol	3	2	1	6
Octan-1-ol	1	1	1	1
				Σ **7**
Instruments				
Energy consumption	< 0.1 kw/h per sample			0
Occupational hazard	Analytical process hermitization			0
Waste	1–10 mL			3
	No treatment			3
				Σ **6**
Total penalty points: 13				
Analytical Eco-Scale score: **87**				

environments (which is already accounted for in item 12), does not contain persistent, bio-accumulative, and toxic (PBT) components exceeding 0.1% according to its safety data sheet.

Although the score of 0.79 was considered reasonable, especially given the method's simplicity and the inherent complexity of essential oil blends, the use of a reduced volume cuvette would decrease the amount of sample required for each analysis. This reduction would minimize waste, contributing to an enhanced overall score. A point of concern that lowers its score is automation where flow injection analysis regime could be used to speed up measurements and automatize but SFME sample preparation could be difficult to produce in-line. However, the authors considered the feasibility of an 'at-line' approach, extracting a partial sample for in situ quality control. While not as green as the other two, it still outperforms the offline approach. Besides, the degree of automation of the method also lowers the score, as it would be necessary one quartz cuvette for each sample to be analyzed, what would significantly increase the cost required to conduct the analysis.

The second metric used in this work was GAPI [33], another calculator that generates a pictogram and uses a three-color system (red, yellow, and green) for greenness assessment (Fig. 5B). This metric does not provide a score for the tested method, but the user can compare it with other methods by the colors attributed to each one of the 15 parameters. For the proposed method, three parameters were left blank since there was no extraction procedure to be applied. Besides, 7 out of 12 applicable parameters received the maximum green score (parameters 2–4, 9, 10, 12 and 13), same quantity as AGREE pictogram (parameters 2, 4, 6, 8–11). As the essential oil blend sample does not need specific storage, preservation, or transport before the analysis, these criteria also received maximum score. GAPI classifies the reagents using the National Fire Protection Association (NFPA) system for health and safety hazard evaluation. In this system, both propan-1-ol and octan-1-ol have an NFPA health score of 1, earning a green rating. However, in terms of safety hazards, despite having an NFPA instability score of 0, their flammability scores are 2 and 3, respectively, placing this hazard in the intermediate category. The only point of concern in the pictogram is the lack of treatment for the waste. However, the options fail to consider the hazardous nature of the waste, indicating a potential area for improvement in this metric. This is significant because certain residues pose greater environmental risks than others, which is not the current scenario.

The Analytical Eco-Scale [32] was also employed to evaluate the method's greenness and verify the compatibility of the results with the first tested metric. The evaluation consists of deducting penalty points from a total of 100 for method's greenness classification. This scale not only considers the quantity of reagents and their toxicity but also their classification according to the Globally Harmonized System of Classification and Labeling of Chemicals (GHS), considering the presence of pictograms and signal words for the assignment of penalty points. Furthermore, it accounts for occupational hazards and waste treatment. Given that, the procedure can be conducted in a fume hood, transferred promptly to a volumetric flask, and that the spectrofluorimeter can be sealed, it was concluded that vapor emission is negligible, and no penalty points were deducted in occupational hazard parameter. A crucial aspect of this method is the absence of waste treatment, given that the sample, consisting of a dispersion of oily and aqueous phases in SFME, cannot be easily separated. Besides that, the method had only 13 penalty points, scoring a total of 87 points according to this metric, classifying it as excellent in terms of green analysis and being in accordance with AGREE and GAPI previous results. A penalty point table based on the original article [32] regarding the proposed method is presented in Table 3.

Overall, all three metrics suggest that the proposed method adopts an environmentally friendly approach despite employing different classification systems.

4. Conclusions

The quality control of complex samples like essential oil blends remains a challenging task that requires attention from the scientific community. In this work, a straightforward and cost-effective method aligned with green analytical chemistry was developed for quantification of sweet orange and lavender EOs contents in blends containing both. The use of SFMEs as sample preparation allowed for a low consumption of EOs, which is economically significant. Additionally, there was an increase in the fluorescence when compared to the essential oil alone. Regarding analytical parameters of merit, the LOD and LOQ obtained were 2.39 μg mL^{-1} and 7.97 μg mL^{-1} (sweet orange EO) and 19.8 μg mL^{-1} and 65.9 μg mL^{-1} (lavender EO). It was possible to quantify up to 0.46% and 3.69% of sweet orange and lavender, respectively, in blends containing both EOs. The recovery values in the 25:75, 50:50 and 75:25 proportions were 103.9 ± 23.8 (CV= 9.22%), 103.1 ± 7.9 (CV= 3.10%) and 94.4 ± 13.7 (CV= 5.78%) respectively, for sweet orange and 100.2 ± 28.7 (CV =11.56%), 97.0 ± 10.7 (CV= 4.44%) and 92.7 ± 7.7 (CV= 3.39%), respectively, for lavender EOs. Greenness assessments were performed using Analytical Eco-Scale, GAPI and AGREE. The first one generated a score of 87 out of 100, and in the second and third metrics, seven out of twelve utilized parameters achieved the highest score, with an AGREE total score of 0.79 out of 1.00. The resulting score for the three metrics along with the acceptable results for the analytical parameters of merit position this approach as both analytically satisfactory and aligned with the principles of green chemistry, besides showing potential to be applied in real quality control scenarios.

CRediT authorship contribution statement

Beatriz Serrão Monteiro Bastos: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Rosana Candida Macedo:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Ricardo Queiroz Aucelio:** Writing – review & editing, Visualization, Supervision, Resources, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendice A

Table A.1
Codes for different volume proportions of sweet orange EO:octan-1-ol and lavender EO:octan-1-ol and their respective concentration in mg mL^{-1}.

Code	EO: octan-1-ol proportion (v/v)	Sweet orange EO (mg mL^{-1})	Lavender EO (mg mL^{-1})
A	1:4	1.72	1.78
B	1:6	1.23	1.27
C	1:9	0.86	0.89
D	1:19	0.43	0.45
E	1:49	0.17	0.19
F	1:499	0.02	0.02

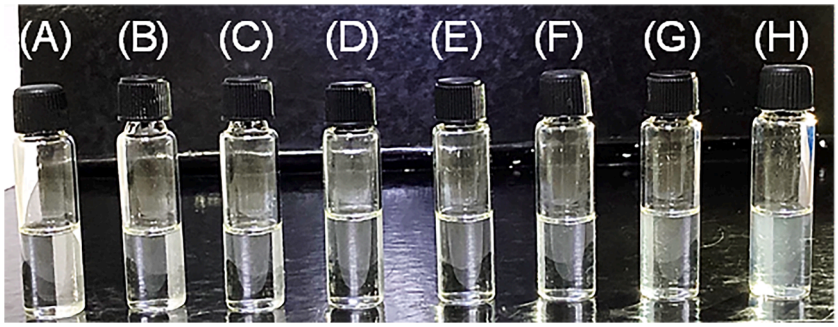

Fig. A.1. SFME systems from sweet orange essential oil containing different amounts of water: (A) 0.0 (B) 0.5 (C) 1.0 (D) 1.5 (E) 2.0 (F) 2.5 (G) 3.0 and (H) 3.5 mL. For each sample, 50 μL of oily phase containing EO diluted in octan-1-ol (1:4 v/v), and propan-1-ol up to 5 mL were used.

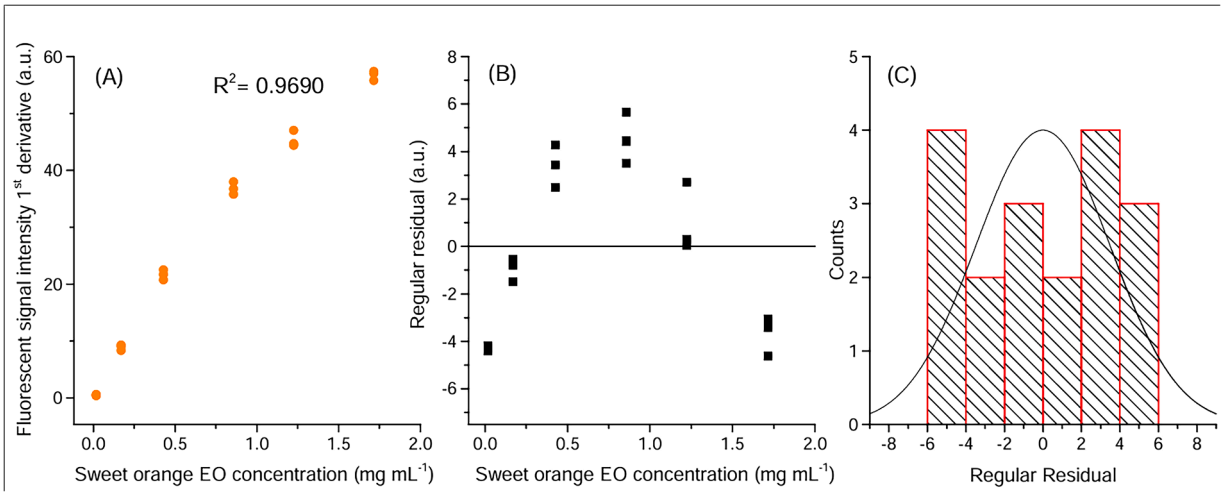

Fig. A.2. (A) Analytical curve based on data obtained for sweet orange analysis without inner filter effect correction. (B). Residual plot and (C) histogram of residual plot are presented to evaluate the linearity of the analytical curve. For analytical curve construction, the fluorescent signal intensities were collected at $\lambda = 316.8$ nm from the first derivative of the synchronous scanning spectrum ($\Delta\lambda = 100$ nm).

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