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Excitation-Emission Matrix Fluorescence Spectroscopy in Hydro-Alcoholic Systems to Discriminate Different Citrus Essential Oils

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Differencing citrus essential oils was achieved by excitation-emission matrix fluorescence spectroscopy using hydro-alcoholic systems with low sample content. Studies were employed to assess the optimal conditions, aiming to encompass all evaluated citrus essential oils (sweet and sour orange, grapefruit, lemon, and tangerine) without compromising measurement reproducibility. In addition to low sample consumption, a significant increase in fluorescence was achieved, which can be explained by the formation of weak micelle-like aggregates in surfactant-free microemulsions. To demonstrate the potential of the proposed method for discriminating samples, fluorescence excitation-emission matrix data were utilized for clustering analysis. Unfolded principal component analysis was successfully applied, with a cumulative variance of 99.5% based on three principal components. Complementary analyses by liquid chromatography were conducted to confirm the relationship between fluorophores and the non-volatile fraction, characteristic of citrus essential oils. The combination of low sample consumption and high-water content makes the application of these systems advantageous for essential oil monitoring also attending requirements for a greener analytical method, as confirmed by an Analytical Eco-Scale score of 85.

Keywords: citrus essential oil, hydro-alcoholic medium, fluorescence spectroscopy, excitation-emission matrix, unfold principal component analysis

Introduction

Citrus essential oils (CEOs) are one of the most commercialized essential oils (EOs) in the world[1] as they have gained popularity because of their biological activities.[2] Currently, many industrial sectors, such as food and beverages, cosmetics, and pharmaceuticals, have a great demand for EOs.[2,3] Because of their high economic importance, it is necessary to invest in methods to guarantee authenticity and traceability. In this matter, gas chromatography (GC) and high-performance liquid chromatography (HPLC) are extensively used.[4,5] Sensory analysis and physicochemical properties are also employed in more simplistic evaluations.[6] Raman[7] and absorption infrared spectroscopies[8] can also be considered. Another alternative, but not fully explored, is using fluorescence spectroscopy, for instance, applying excitation-emission matrix (EEM) to obtain fingerprinting patterns.

EEM fluorescence spectroscopy is a method used for complex samples, first described by Johnson *et al.*[9] in 1977, and widely applied in dissolved organic matter and petroleum characterizations.[10,11] The main advantage of EEM fluorescence spectroscopy is its selectivity, simplicity, and cost-benefit. New applications in food quality control and authenticity analysis, especially for vegetable oils, have been reported,[12,13] but only a few studies have employed EEM fluorescence spectroscopy on EOs.[14-17] While possessing great potential, it remains relatively unexplored due to practical implications, such as the relatively large EO amounts required in batch analysis, combined with the need for replicates for mathematical treatment. Furthermore, EOs are hydrophobic and present a strong odor, requiring meticulous cleaning to prevent cross-contamination, especially when using mini cells.[14] The extended analysis time should also be considered. Besides, some EOs are susceptible to photobleaching.[15] Finally, the presence of

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numerous chromophores in its composition must also be considered (internal filter effect), necessitating spectral corrections.[18]

An alternative, recently proposed by Macedo *et al*.,[17] and Bastos *et al*.,[19] is the use of hydro-alcoholic systems, also known as surfactant-free microemulsions (SFME).[20,21] In SFMEs, short-chain alcohols are used to stabilize biphasic mixtures (water and oil), forming optically clear dispersions through the formation of weak micelle-like aggregates. Compared to surfactant-based microemulsions (SBME), the SFMEs have a higher water content in their composition.[22] Micellar aggregates result in microenvironments that encapsulate fluorophores, imposing movement and vibration restrictions that minimize the probability of non-radiative decay processes, consequently enhancing the inherent fluorescence of some of the components in EOs.[23] As reported by Macedo *et al*.,[17] these systems were able to enhance spectral detailing when applied to the analysis of sweet orange EO, being useful for fingerprinting. Compared with these previous studies,[17,19] the present work demonstrates the feasibility of using markedly smaller sample volumes and predominantly aqueous systems without compromising fluorescence intensity or discrimination performance. Additionally, the approach was extended to other CEOs, broadening the applicability of the method.

New surfactant-free organized systems were evaluated to achieve better analytical performance in EEM spectrofluorimetry for CEOs aiming at sample discrimination among different *Citrus* species, including grapefruit (*Citrus paradise*), lemon (*Citrus limonum*), sour orange (*Citrus aurantium*), sweet orange (*Citrus sinensis*), and tangerine (*Citrus reticulate*). In this work, an exploratory analysis was also performed to investigate the origin of the fluorescence exhibited by the studied systems, providing further insight into the behavior of fluorophores in SFME environment. Furthermore, these systems can serve as a versatile platform for diverse analytical applications, providing a foundation for rapid and environmentally friendly strategies in EOs research.

In addition to its analytical performance, the proposed approach was quantitatively evaluated using the Analytical Eco-Scale metric.[24] This tool provides an objective assessment of the greenness of the method by assigning penalty points based on reagent hazards, energy consumption, occupational risks, and waste generation. The incorporation of this evaluation reinforces the sustainable character of the method, and highlights its potential as a model for environmentally friendly analytical approaches in essential oil research and beyond.

Experimental

Reagents and chemicals

Commercial grapefruit, lemon, sour orange, sweet orange, and tangerine EOs (100% pure, from the same Brazilian brand) were obtained locally. Hydro-alcoholic systems were prepared using ultra-pure water (Milli-Q gradient A10, Milipore, USA), octan-1-ol (HPLC grade) and propan-1-ol (HPLC grade). Acetonitrile (HPLC grade) was used for chromatographic analyses. A mixture containing 2*H*-chromen-2-one, heptamethoxyflavone, nobiletin, sinensetin (all with 99.99% purity), tetra-*O*-methyl-scutellarein (98.0% purity) and tangeretin (95.0% purity) at 100 mg mL^{-1}, in ethanol (HPLC grade), was also used.

Instrumentation and apparatus

Fluorescence and absorbance measurements were made using the LS-55 (PerkinElmer, UK) and Cary-100 (Varian, Australia) spectrophotometers, respectively. HPLC analyses were performed on Agilent 1200 series (Agilent Technologies, Japan) with diode array photometric absorption and fluorescence detectors. Detailed information on the parameters used for data acquisition and analysis is provided in the corresponding sections of this article. Samples were centrifuged in a BE 4000 Brushless centrifuge (Bio-Eng, Brazil). polytetrafluoroethylene (PTFE) syringe filters (0.45 µm, Whatman, UK) were used to filter samples before HPLC analyses.

Software programs

The FL WinLab software (4.00.02 version, PerkinElmer, USA), Cary WinUV software (3.00(182) version, Varian, Australia), and ChemStation software (2.3.54 version, Agilent, Japan) were used to control the instruments (luminescence and absorbance spectrophotometers and liquid chromatography system, respectively) and collect data. The OriginPro software (version 2022b, OriginLab Corporation, USA) was employed to unfold the spectral data into a two-dimensional matrix and to assemble figures (except for the contour surfaces). RStudio (2025.09.1 version, Posit Software, USA) was used for data pre-processing (eemR and stardom packages) and chemometric treatment (pca3D and ggplot2 packages).

Hydro-alcoholic systems preparation and evaluation

In 25 mL volumetric flasks, 19 mL of ultrapure water and 15 µL of the oily phase, consisting of EO diluted in octan-1-ol

(1:2, v/v), were added. The final volume was adjusted with propan-1-ol, followed by manual agitation. An analytical blank with the same SFME composition, containing only octan-1-ol and no EO, was also prepared to enable spectral background correction (blank subtraction) and ensure accurate fluorescence measurements. Additionally, systems were monitored for three months (stored at 4 and 25 °C but measured at room-temperature) to evaluate the stability. Visual inspections were performed daily during the first 15 working days and then weekly to check for phase separation or turbidity. Thermodynamic stability was also evaluated by centrifugation (1080 relative centrifugal forc (RFC), 20 min). The preparation can theoretically be scaled down to 5 mL volumetric flasks by proportionally adjusting the component volumes, which would reduce the required amount of propan-1-ol to less than 1.5 mL and further enhance the greenness of the method.

Spectroscopy analysis and data pre-processing

For the optimization experiments, the fluorescent signal intensities were acquired at 336/436 nm (excitation and emission wavelengths, $\lambda_{ex}/\lambda_{em}$). Fluorescence emission spectra (360-500 nm) were obtained with excitation at 336 nm, with 10 nm spectral bandpass (excitation and emission) and 1500 nm min^{-1} scan rate. To evaluate the inner filter effect,[18] absorbance spectra (300-600 nm) were acquired at a scan rate of 300 nm min^{-1}, with a data interval of 0.5 nm.

After choosing the optimal formation condition, SFMEs systems were reproduced for each evaluated CEO and analyzed by EEM fluorescence spectroscopy using a 3 mL quartz cuvette. Excitation spectra (200-650 nm, steps of 10 nm) were recorded for emission wavelengths (280-700 nm) with increasing steps of 0.5 nm, with 10 nm spectral band passes, and 1500 nm min^{-1} scan rate for the emission monochromator. For sweet orange EO, a 50% transmittance (nominal value) neutral density filter (Newport, USA) was used. For the others, a 1% transmittance (nominal value) neutral density filter (Newport) was required. Raw data were obtained in arbitrary units (a.u.). Data pre-treatments included blank subtraction and scattering removal.

For chemometric treatment, 10 replicates of each sample were analyzed. To reduce time *per* analysis, a restricted excitation (200-400 nm) and emission (340-550 nm) wavelength ranges were used. In this case, data pre-treatment also included Raman unit (R.u.) conversion to normalize information.[25] For the R.u. conversion, measurements were performed in triplicate using ultrapure water over the full ranges of 200-50 nm (excitation, 10 nm steps) and 280-700 nm (emission, 0.5 nm steps).

Chemometrics

Chemometrics was applied for discriminant analysis, using unfold principal component analysis (UPCA). All EEM data, after pre-processing, were unfolded into a two-dimensional matrix, and a principal component analysis (PCA) was performed with variables centered and scaled for better accuracy. Principal components (PCs) were used to discriminate the CEOs in five groups (grapefruit, lemon, sour orange, sweet orange, and tangerine). As the observed fluorescence originates mainly from the non-volatile fraction (as further detailed in the following topic and in the Results and Discussion section of this work), which contains citrus-specific compounds, this makes the approach particularly suitable for differentiation purposes, and an exploratory analysis using UPCA was sufficient to reveal intrinsic groupings and patterns. More complex supervised methods were not required for the objectives of the current work.

Investigation of fluorophores

HPLC analysis was used to corroborate the hypothesis that the response obtained by the spectrofluorimetric analysis originated from compounds present in the non-volatile fraction of CEOs. Chromatographic analyses were made using a Zorbax Eclipse XBD-C18 column (250 × 4.6 mm, 5.0 µm) from Agilent Technologies (USA) at 30 °C with 5.0 µL sample introduction volume. Ultrapure water (A) and acetonitrile (B) were used as mobile phase with a linear gradient (0-7 min, 50% B; 7-13 min, 50-60% B; 13-15 min, 60-80% B; 15-17 min, 80% B, 17-20 min, 80-100% B; 20-25 min, 0% B; 25-27 min, 100-50% B, 27-30 min, 50% B) and flow rate of 1.0 mL min^{-1}. Chromatograms were acquired using diode array photometric absorption (210-400 nm, at 330 nm), and fluorescence (336/436 nm, $\lambda_{ex}/\lambda_{em}$) detectors. In accordance with the specificity of this technique, new conditions were established for the SFME systems: 60 µL of a mixture containing EO and octan-1-ol (1:2, volume proportion), 2 mL of ultrapure water, and propan-1-ol up to 5 mL.

Greenness assessment by the Analytical Eco-Scale

The greenness of the proposed method was evaluated using the Analytical Eco-Scale,[24] which assigns an initial score of 100 points from which penalty points (PPs) are subtracted according to reagent consumption, waste generation, energy demand, and safety or environmental hazards. The final Eco-Scale score was obtained by subtracting the total PPs from 100, and methods were

classified as excellent (> 75), acceptable (50-75), or inadequate (< 50) from the green chemistry perspective.

Results and Discussion

Hydro-alcoholic systems have been investigated as versatile media for enhancing fluorescence responses of EOs, due to their ability to form weak micelle-like aggregates.[17,19] In the present work, the chosen condition for the hydro-alcoholic systems was adapted based on the previous studies. In the study conducted by Macedo *et al*.,[17] it was concluded that SFME, formed through pseudo-ternary liquid mixtures (utilizing sweet orange EO and octan-1-ol as the oily phase, along with water and propan-1-ol), significantly increased fluorescence measured from EOs, making these systems useful for obtaining fingerprinting patterns. Subsequently, the same research group[19] demonstrated that by using lower proportions of the oily phase, a significant increase in fluorescence signal was obtained for sweet orange EO, especially at 336/436 nm ($\lambda_{ex}/\lambda_{em}$). Based on these considerations and aiming to achieve stable systems with lower sample and higher water contents, in this study a univariate optimization was carried out by monitoring systems with reduced levels of oily phase (composed of sweet orange EO and octan-1-ol, 1:2, v/v). Hydro-alcoholic systems containing different volumes of oily phase (from 5 to 300 µL), water (with volume set at 1.0 mL), and propan-1-ol (to complete a final volume of 5.0 mL) were monitored by fluorescence spectroscopy at 336/436 nm ($\lambda_{ex}/\lambda_{em}$).

Considering the volumes of the oil phase, the results (Figure 1a) showed that fluorescence intensity increased up to 125 µL, with only minor increases in signal intensity up to 225 µL. The increase in fluorescence with oil phase volume is expected due to the higher concentration of fluorophores. However, for larger volumes, the incremental rate of fluorescence decreases, possibly due to the presence of chromophores at higher concentrations, which enhance internal filter effects and light scattering. At low volumes (up to 15 µL), the increase in fluorescence is approximately linear (Figure 1b), indicating that counteracting effects are minimal. Despite the very small sample volumes, fluorescence is still observable, mainly due to the formation of weak micelle-like aggregates in the SFME systems. These aggregates restrict molecular motion, decreasing the likelihood of non-radiative decay and reducing collisional quenching of the fluorophores, thereby maintaining high signal intensity. This behavior aligns with molecular confinement theory, in which restricted molecular dynamics within organized microenvironments, such as those formed by weak micellar aggregates, limit vibrational relaxation pathways and favor radiative decay, enhancing emission efficiency.[23] To further confirm the influence of chromophore effects at higher oil phase volumes, absorption spectra were obtained for each condition (Figure 1c), which confirmed that at 436 nm (the wavelength used for fluorescence measurements), the internal filter effect only becomes significant above 15 µL, explaining the linear correlation observed in Figure 1b. Working at oil phase volumes above 15 µL would require additional spectral corrections due to the increasing influence of internal filter effects, adding complexity to the analysis. Based on these observations, 15 µL of oil phase were selected for subsequent experiments, ensuring both high fluorescence and medium transparency.

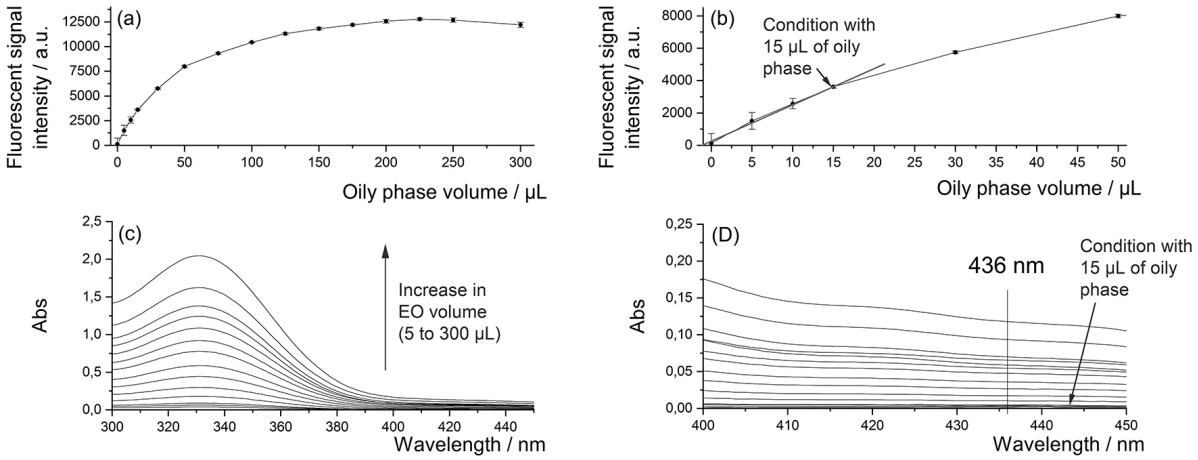

Figure 1. Optimization for adjusting the volume of the oily phase (sweet orange EO and octan-1-ol, 1:2 v/v): (a) fluorescent signal intensity (336/436 nm, $\lambda_{ex}/\lambda_{em}$) in function of different oily phase volumes (0 to 300 µL) and (b) zoom in on volumes between 0 and 15 µL to highlight the linear range. (c) Absorbance (300 to 450 nm) and (d) zoom in the 400 to 450 nm range to evaluate inner filter effect for sample systems with emphasis on the response obtained for the system containing 15 µL of oily phase. A fixed volume of water (1.0 mL) and propan-1-ol (completing final volume up to 5 mL) were used to form homogeneous hydro-alcoholic systems.

After establishing the ideal oily phase volume for sweet orange EO (5 μL in a 15 μL total oily phase), this system was successfully reproduced for the other CEOs (grapefruit, lemon, sour orange, and tangerine). Subsequent fluorescence spectroscopy analysis revealed that all these other evaluated varieties exhibited a superior fluorescence response compared to the system comprising sweet orange EO. In fact, for grapefruit EO, the signal was so intense that even the optical density filter of 1% transmittance was not capable of adjusting fluorescence under the detector saturation point. However, as observed in Figure 1b, higher signal variations (based on replicate study and reflected in the error bars) were obtained for systems with smaller volumes of oily phase. This fact can be attributed to difficulties in reproduce systems when sampling lower volumes of the oily phase due to nature of the oil samples.

To find a compromise between the EO sample content and the fluorescence intensity characteristic from all the analyzed samples, a further study was made aiming to find a proper dilution factor for the system, finding a single preparation condition for all. In this sense, the SFME systems prepared with 15 μL of oily phase (sweet orange EO and octan-1-ol, 1:2 v/v) and 1 mL of water were further adjusted, with propan-1-ol, to the final volumes: 10, 25, and 50 mL (Figure 2a). At 25 mL final volume, the fluorescence from sweet orange EO could still be measured, leaving room to observe fluorescence from the other EOs within the range before detector saturation. The complement to this study was made aiming to replace part of the propan-1-ol content by water, enabling signal improvement up to the limit of visual transparency of the system. In Figure 2b, fluorescence measured from orange EO SFME systems containing 5, 10, and 19 mL of water (respectively containing 20, 15 and 6 mL of propan-1-ol as complement) are shown. Above 19 mL of water, system became visually turbid showing that this was the limit for the water content. Finally, a study was made using only sweet orange EO as oily phase (5 μL) ignoring the prior dilution with octan-1-ol but using the water and propan-1-ol proportions set in the prior experiments. In agreement with the results obtained by Macedo *et al.*,[17] the formation of SFMEs did not occur (Figure S1, Supplementary Information (SI) section).

Taking into consideration the high fluorescence measured from some of CEOs (especially grapefruit), the compromise condition among sample amount, signal intensity from all of CEOs to adjust response under the saturation limit of the detector and reproducibility of results was found to be appropriate, using 15 μL of the oily phase (mixture containing EO diluted in octan-1-ol at 1:2, v/v), 19 mL of ultrapure water, and propan-1-ol until volume adjustment to 25 mL. Systems stability was evaluated for all CEOs in such conditions (Figure S2, SI section) and there was no evidence of visual alteration, such as phase separation and turbidity, during sample monitoring (three months at 4 and 25 °C), also after centrifugation test (1080 RFC for 20 min). These results indicated thermodynamic stability, which is characteristic of SFMEs.[20]

Following the univariate optimization and establishment of the compromise condition, the hydro-alcoholic systems of each CEO were evaluated by EEM fluorescence spectroscopy. Results revealed that all CEOs included in the study exhibited significant fluorescence intensity values in the same region as sweet orange EO (at 336/436 nm, $\lambda_{ex}/\lambda_{em}$). Additional experiments with non-citrus EOs were also conducted, and none of them exhibited maximum fluorescence emission in the same region, indicating that this could be a characteristic response of compounds present in CEOs. In the light of this, samples were then replicated, and the results of the EEM fluorescence spectroscopy analyses were processed to assess the feasibility of applying these systems for the differentiation of CEOs.

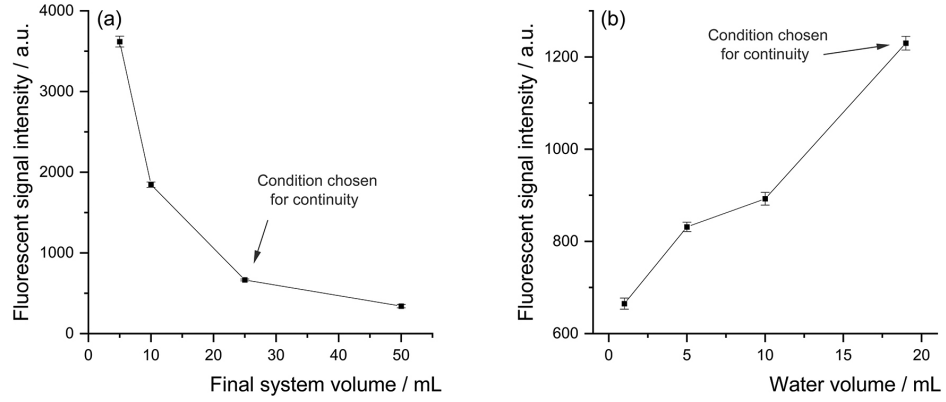

Figure 2. (a) Evaluation of the final volume of the hydro-alcoholic systems using a fixed volume (1.0 mL) of water and 15 μL oily phase (5 μL of sweet orange EO and 10 μL of octan-1-ol) using propan-1-ol to adjust the final volume. (b) Fluorescence intensity from diluted systems containing 15 μL of oily phase as a function of different water volumes, adjusting final volume to 25 mL using propan-1-ol.

Initially, an extended wavelength range (200-650 nm and 280-700 nm for excitation and emission, respectively) was used to verify the overall spectral response (Figure S3, SI section). Then, it was decided to restrict the wavelength ranges (200-400 nm and 340-550 nm for excitation and emission, respectively), in order to enhance the region of greater spectral detail, also reducing total analysis time (from 26 to 5 min) and simplifying data matrix to be used in the chemometric treatment (from 46 × 840 to 21 × 420). Data pre-processing was carried out to minimize the instrumental variation effects and using Raman unit (R.u.) conversion.[25] The fingerprint patterns obtained for each CEO in hydro-alcoholic system after wavelength range adjustment, and data pre-treatment are present in Figure 3. Despite the similarity of the obtained spectra, in terms of the maximum excitation and emission wavelengths, it was possible to notice that they present differences in the pattern of the fluorescence intensities observed for each EO after EEM fluorescence spectroscopy analysis. To prove the potential of application of these systems to obtain fingerprint patterns useful for the differentiation of CEOs, UPCA was applied as chemometric approach. As a result, the three first principal components (PCs) were sufficient to explain 99.5% of the variances (73.3, 20.2 and 6.0% for PC1, PC2 and PC3 respectively). Two-dimensional score plots with data distribution for each replicate are shown in Figures 4a-4c.

For UPCA clustering analysis, CEOs were grouped based on their species. In the first plot (Figure 4a), each type of EO occupies a different quadrant, except for sweet and sour orange EOs. In the sequence, the eigenvectors were decomposed to examine which pairs of excitation and emission wavelengths ($\lambda_{exc}/\lambda_{em}$) exert influence on each principal component (considering PC1, PC2, and PC3). Results are shown in Figure 5. It was found that 336 and 436 nm ($\lambda_{exc}/\lambda_{em}$) proved to be relevant for the effective differentiation of various CEOs. Considering the excitation wavelength, the region bellow 250 nm is important for PC3. However, this region exhibits a significant spectral variation in the analyses conducted with sweet orange EO (Figure S4, SI section), which may explain the high variability observed among their replicates, especially when PC3 is taken into consideration (Figures 4c and 2c).

Regarding the observed fluorescence, it is believed to originate mainly from compounds of the non-volatile

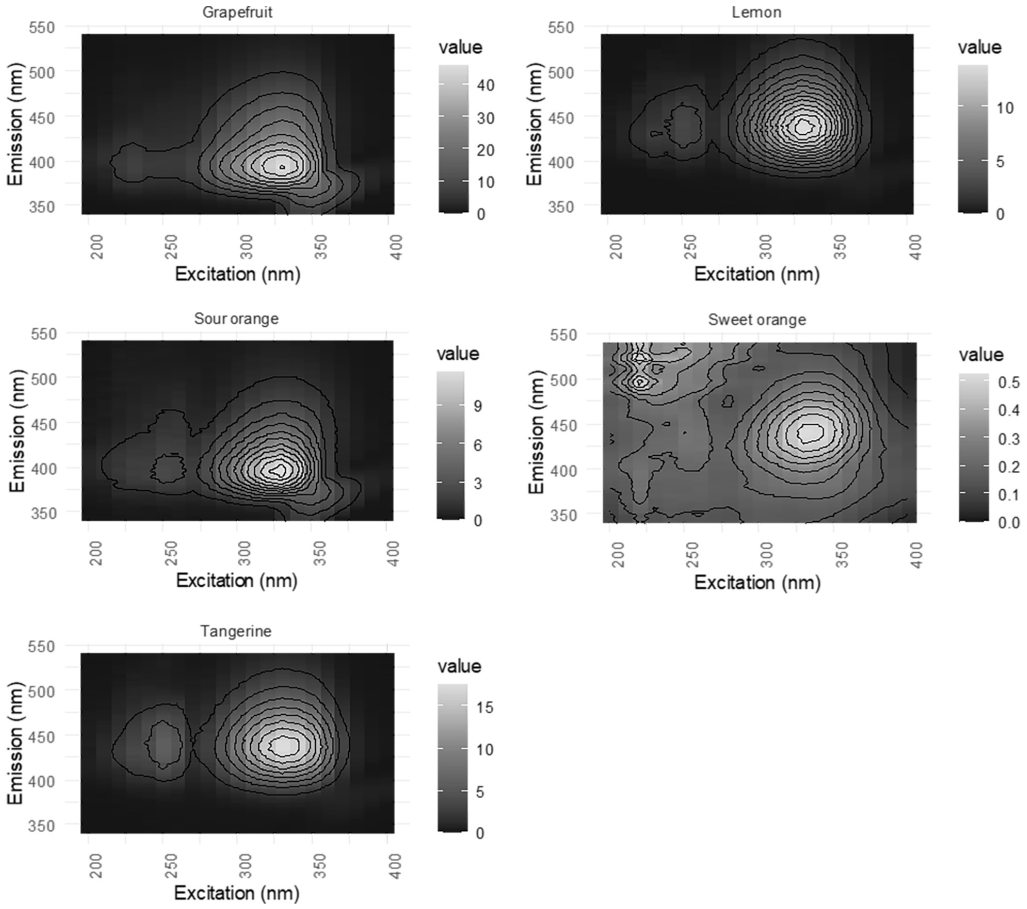

Figure 3. EEM contour obtained for CEOs in SFME media in a restricted wavelength range after scattering removing and blank subtraction. Intensity fluorescence in Raman units (R.u.). Dilution factor was not considered.

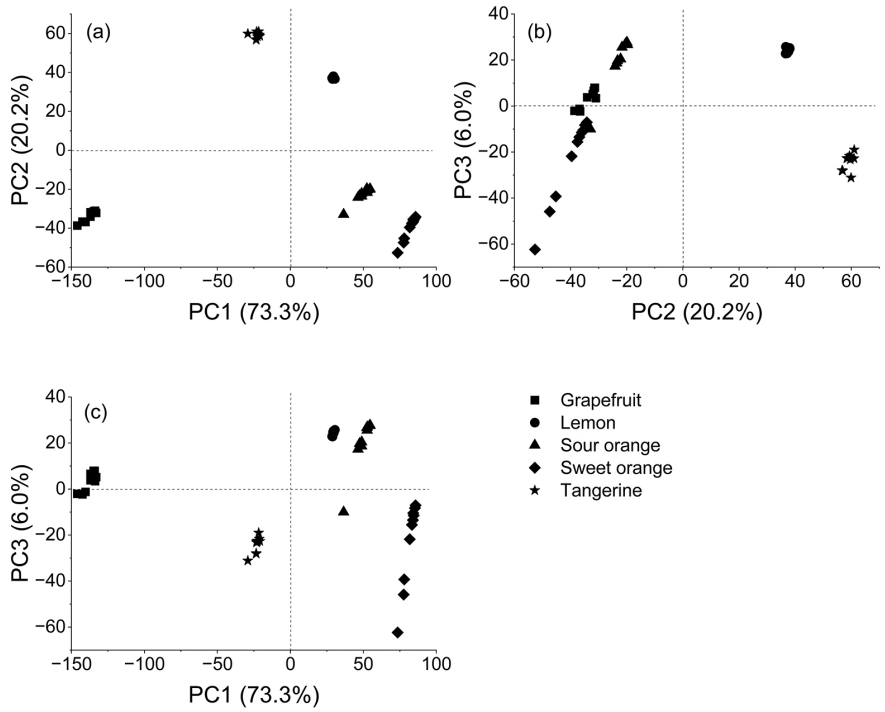

Figure 4. Two-dimensional scores plots for the citrus essential oils evaluated by EEM fluorescence spectroscopy in SFME media after UPCA treatment: (a) PC1 × PC2 (cumulative variance: 93.3%), (b) PC2 × PC3 (cumulative variance: 26.2%), (c) PC1 × PC3 (cumulative variance: 79.5%).

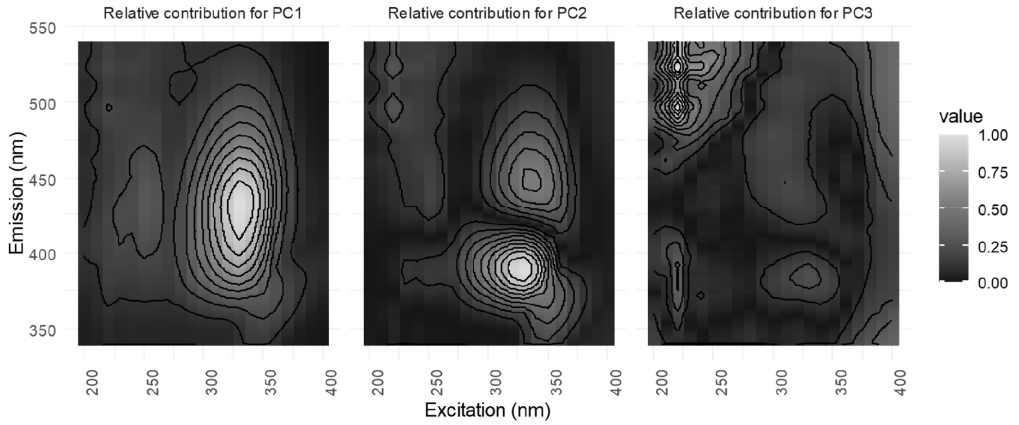

Figure 5. Contour surface with the relative contribution of each excitation and emission wavelengths for the three principal components (PC1, PC2 and PC3) plotted as contour surfaces.

fraction. Commercial CEOs are usually obtained by fruit peels cold pressing, consequently, they have a particular characteristic, that is the presence of a fraction containing non-volatile compounds. Primordially composed of coumarins, psoralens, and polymethoxyflavones (Figure S5, SI section), this fraction can be considered important for the authenticity of CEOs due to its less complex composition.[26] Fluorescence in the wavelength regions used here, associated with compounds of the non-volatile fraction, has been reported for related classes of compounds in the literature, thereby supporting this hypothesis.[27,28] In 2000, Izquierdo *et al.*[27] demonstrated the effectiveness of applying spectrofluorimetry in determining coumarins (umbelliferone, scopoletin, and 4-methylumbelliferone) in distilled beverages in a hydro-alcoholic medium. In this study, the group used similar wavelength values (340 and 425 nm). In 2013, Hroboňová *et al.*[28] determined herniarin, compound of the coumarin class, in propolis (using ethanol as diluent) by HPLC (with fluorescence detector) operating at 320/450 nm, with limits of quantification 20,000 times smaller than those obtained using UV detection (at 280 nm).

To evaluate if the fluorophores observed on 3D spectra (Figure 3) are non-volatile compounds, complementary analyses were performed by HPLC. Chromatographic conditions were adapted from previous studies,[29,30] using hydro-alcoholic media as a sample preparation approach.

Chromatograms were monitored with both a fluorescence detector (FD), to identify the fluorophores responsible for the intense signal at 336/436 nm, and a diode array detector (DAD), which provided absorption spectra of compounds eluting at corresponding retention times. Since coumarins, psoralens, and polymethoxyflavones (Figure S5, SI section) possess characteristic UV-Vis absorption spectra.[26] The combined use of FD and DAD allowed the identification of peaks corresponding to these compound classes, with the main objective of relating the observed fluorescence signals to the non-volatile fraction of CEOs. Results (Figures 6a-6e) showed numerous fluorophores capable of fluorescence in the region of interest. The absorption spectra of the peaks with retention times matching the highest intensity fluorescence peaks (Figures 6b-6e) were evaluated (Figure S6, SI section), confirming characteristic coumarin spectra for all marked compounds.

For sweet orange EOs, low absorbances were obtained, which did not permit an effective comparison. Compared to other CEOs, sweet orange EO has a less complex non-volatile fraction, primarily composed of polymethoxyflavones.[26] To investigate if these substances were responsible for the observed fluorescence, a mixture containing five polymethoxyflavones (heptamethoxyflavone, nobiletin, sinensetin, scutellarein, and tangeretin) and chromen-2-one as internal standard was also used for chromatographic comparison (Figure 7). As a result, all polymethoxyflavones exhibited signals in the monitored region (336/436 nm, $\lambda_{ex}/\lambda_{em}$), corroborating the influence of the non-volatile fraction on the results found for sweet orange EO after spectrofluorimetry analysis. These results corroborate the correlation between the fluorescence signal and the non-volatile fraction. The non-volatile fraction is increasingly recognized as an important marker of authenticity in citrus essential oils.[26,31] Confirming this relationship positions this approach as a promising alternative for the development of new methods for rapid quality assessment, adulteration detection, and evaluation of complex blends in citrus essential oils.

Finally, the greenness assessment was carried out using the Analytical Eco-Scale method,[24] which was selected because it provides a straightforward and comprehensive evaluation of reagent consumption, solvent hazards, sample preparation steps, and waste generation. This metric was considered the most appropriate for the present study, as it specifically addresses the impact of the solvents and preparation conditions employed in the proposed method, allowing a consistent comparison with green chemistry principles. The detailed penalty points assigned to each reagent are summarized in Table 1, and the overall

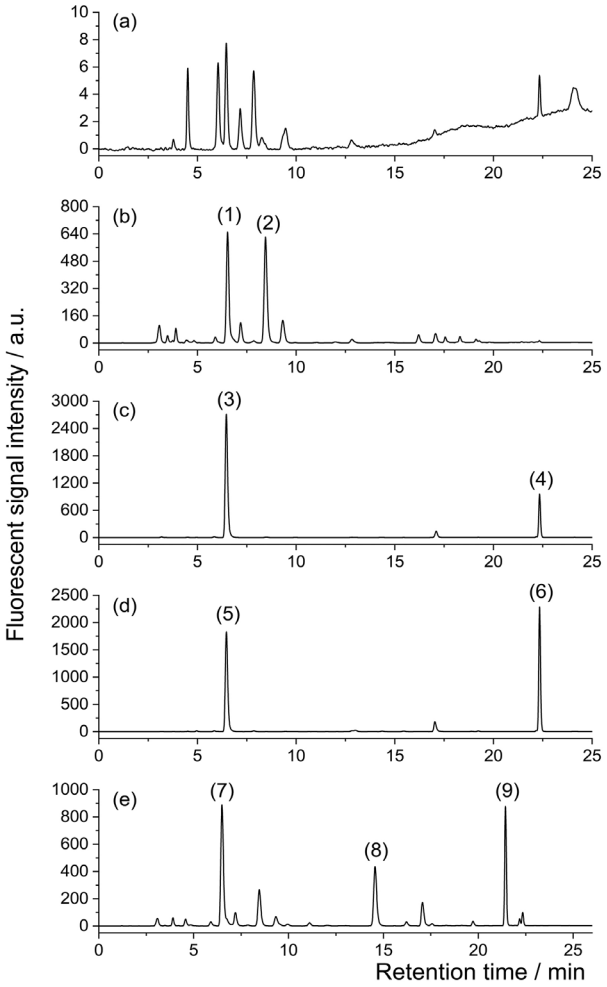

Figure 6. Chromatograms obtained for (a) sweet orange, (b) sour orange, (c) lemon, (d) tangerine, and (e) grapefruit essential oils in hydro-alcoholic system. Chromatograms were acquired using fluorescence detector (336/436 nm, $\lambda_{ex}/\lambda_{em}$). Peaks (1-9) correspond to the most intense signals. Their UV spectra is provided in the Supplementary Information section (Figure S6).

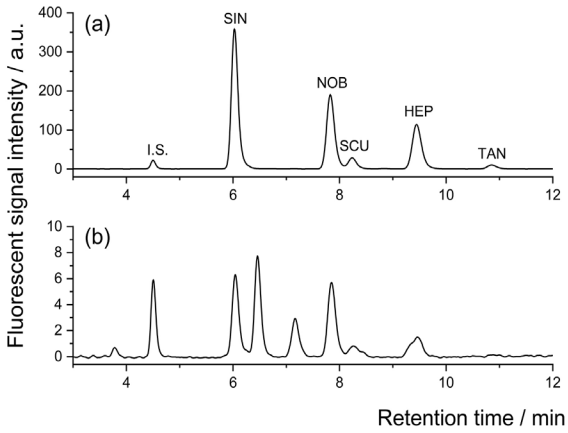

Figure 7. (a) Chromatograms of a mixture containing chromen-2-one as internal standard (I.S.) and five polymethoxyflavones: sinensetin (SIN), nobiletin (NOB), scutellarein (SCU), heptamethoxyflavone (HEP), and tangeretin (TAN), at 100 mg mL^{-1}. (b) Chromatograms of sweet orange EO and chromen-2-one as I.S. in hydro-alcoholic system. Chromatograms were acquired using fluorescence detector (336/436 nm, $\lambda_{ex}/\lambda_{em}$).

Table 1. Penalty points (PPs) for the proposed method, calculated according to the Analytical Eco-Scale metric[24]

Reagents[a]	Sub-total PP	Total PP
Octan-1-ol	amount (< 10 mL) = 1 GHS pictograms = 1 signal word (warning) = 1	1 × 1 × 1 = 1
Propan-1-ol	amount (< 10 mL) = 1 GHS pictograms = 3 signal word (danger) = 2	1 × 3 × 2 = 6
Ultrapure water	amount (10-100 mL) = 2 GHS pictograms = 0 no signal word = 0	2 × 0 × 0 = 0
		Σ 7
Instruments	Sub-total PP	Total PP
Energy consumption	spectrofluorimetry (< 0.1 kWh *per* sample) = 0	0
Occupational hazard	analytical process hermitization = 0	0
Waste	amount (> 10 mL) = 5	5
	no treatment = 3	3
		Σ 8
	Total penalty points: 15	
	Analytical Eco-Scale total score: 85	

[a]For reagents, the total PP was calculated as the product of the amount PP and the hazard PP. The amount PP was set to 1 for volumes lower than 10 mL and 2 for volumes between 10 and 100 mL. The hazard PP was defined as the number of GHS (Globally Harmonized System) pictograms multiplied by the signal word factor (warning = 1, danger = 2).

Eco-Scale score was then calculated, resulting in a score of 85. This value indicates that the proposed method can be classified as an excellent green analytical procedure. It is important to indicate that the final volume of this system could be easily re-scaled to 5.0 mL by adjusting volumes of components keeping the previously established proportions.

Conclusions

This study aimed to assess the potential of excitation-emission matrix fluorescence spectroscopy in hydro-alcoholic systems for discriminating citrus essential oils. Following univariate optimization, a compromise condition was established, enabling reproducible analysis with minimal sample and solvent consumption. Under these conditions, clear differentiation among CEOs (sweet and sour orange, grapefruit, lemon, and tangerine) was achieved by UPCA, with a cumulative explained variance of 99.5% for the first three PCs (73.3, 20.2, and 6.0%). Eigenvector decomposition revealed a significant contribution at 336/436 nm ($\lambda_{ex}/\lambda_{em}$), and complementary HPLC analyses supported the hypothesis that this common fluorescence signal arises from fluorophores in the non-volatile fraction. The low sample consumption, high water content, transparency, and low viscosity of these systems highlight their practical advantages. Furthermore, the greenness assessment using the Analytical Eco-Scale resulted in a score of 85, indicating that the proposed method can be classified as an excellent green analytical procedure. Altogether, the findings demonstrate that hydro-alcoholic fluorescence systems provide a simple, sustainable, and reliable approach for CEO monitoring, with promising applications in quality control and authenticity assessment.

Compared to our previous studies, this work extends the approach to multiple citrus species, incorporates univariate optimization for improved reproducibility, and confirms the contribution of the non-volatile fraction to the observed fluorescence. In addition, the use of surfactant-free microemulsions with high water content allows minimal consumption of organic solvents and essential oil samples, while enhancing the fluorescence response. These features make the systems versatile for various analytical applications, offering a sustainable and rapid platform for essential oil quality control and authenticity monitoring. The proposed method is also promising for broader applications, such as the monitoring of sample adulteration and the differentiation of oils according to their geographical origin, reinforcing its potential for comprehensive authenticity assessment in essential oil research.

Supplementary Information

Supplementary information is available free of charge at http://jbcs.sbq.org.br as PDF file.

Data Availability Statement

All data supporting the findings of this study are available within the article and its supplementary information.

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Author Contributions

R.C.M. was responsible for conceptualization, investigation, methodology, formal analysis, software, data curation, writing original draft, writing review and editing, and visualization; A.L.M.C.C. for supervision and visualization; R.Q.A. for funding acquisition, supervision, writing review and editing, and visualization.

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