A Comparative Study Using UV-Vis, NIR, and FTIR Spectral Fingerprinting in Yerba Mate Leaves through Mixture Design Extractions and ASCA Models

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Analysis of variance (ANOVA)-simultaneous component analysis (ASCA) is a method of choice for factorial design studies of environmental impacts on plant metabolomes and can be used to quantitatively carry out this comparative analysis. The impacts of seven mixture design extractor systems made up of ethanol, dichloromethane, and hexane and their 1:1 binary and 1:1:1 ternary solvents for several replicate experiments (n = 3, 4, 5) were assessed using ASCA models determined from Fourier-transform infrared (FTIR), near-infrared (NIR), and UV-Vis spectroscopic measurements on yerba mate leaf extracts. This analysis considered two-factor effects: secondary sexual dimorphism (male and female plants) and cultivation systems (monoculture and agroforestry), as well as their interaction effect. The three binary solvents were found to be more efficient extractor systems for all four detectors as they found 83 main and interaction effects significant at or above the 95% confidence level compared with only 47 for the pure solvent extracts. Binary solvent extracts resulted in averages between 44.04 and 86.61% for the ASCA total effect variances compared with 40.62 to 71.07% for pure extractors. Of the 60 significant effects found for experiments with 5 repetitions 53 or 88% were obtained with only triplicate determinations. The choice of spectroscopic technique and solvent system have large impacts on metabolomic analysis results.

Keywords: cultivation system, FTIR, NIR, mixture design, secondary sexual dimorphism, UV-Vis spectroscopy

Introduction

Ilex paraguariensis A. St. Hill. or yerba mate, is a tree species native from South America. Its leaves and thin green branches are widely used for various beverage preparations. Due to its pharmacological properties, yerba mate is becoming increasingly important as a health-promoting plant species known for its antioxidant, anti-inflammatory, antimutagenic, and anti-obesity functions.[1] Yerba mate is a dioecious tree, showing structural, physiological, and chemical sexual dimorphism.[2] This means that trees of two genders are responding differently to various environmental constraints, differently adjusting the plant architecture, leaf, and plant photosynthesis, which has implications on the primary and secondary leaf metabolites,[3] and finally on biomass formation.[4] It reaches a height of 15 m in its natural habitats, the second stratum of subtropical rainforest with the *Araucaria angustifolia* as a dominant species. This means that yerba mate grows naturally in shaded environments.[4] Yerba mate is today rarely grown in agroforestry, a system that mimics some characteristics of primary forests and generates greater ecological sustainability, but more frequently in a monocultural system with only one plant species grown in a sunnier cultivation environment that also allows easier agricultural management.[4]

Analyzing secondary metabolites requires specific analytical techniques to assess the chemical variations involved. Spectroscopic techniques are widely used and

can provide valuable data in this context. They have been successfully applied for monitoring metabolites in plants cultivated under varying climatic and environmental conditions.[5-8] For example, Fourier-transform infrared (FTIR) spectroscopy offers a fast, cost-effective, and non-destructive way to obtain qualitative and quantitative information.[9] It relies on the interaction of vibrating functional groups in molecules with infrared light, resulting in predictable characteristic patterns.[10] Another valuable technique used in plant metabolic assessment is near-infrared (NIR) spectroscopy, which comprises low-frequency radiation adjacent to the red hues in the visible spectrum.[11] In the NIR region, characteristic absorbed radiation is often attributed to different chemical bonds, such as C–H, N–H, S–H, C=O, and O–H, present in the sample.[12] This radiation region is also used to assess the environmental effects on plant metabolic profiles.[13-16] Ultraviolet and visible spectroscopy (UV-Vis) can be applied to plant metabolic analysis as this technique is based on light absorption promoting electrons in highly delocalized molecular orbitals.[17] In this way, it is possible to investigate metabolic responses of plants in their growth environments.[18,19] Spectral techniques provide a large and complex set of data, due to the large number of plant components,[20] suggesting that their analyses must use diverse strategies, considering that these data contain many more variables than samples.

Chemometric tools can be used to interpret these spectral data as a way of analyzing the significance of multi-level factors experienced in the field on the plant metabolome.[21] In this context, the analysis of variance (ANOVA)-simultaneous component analysis (ASCA) method deals with multivariate datasets containing an underlying experimental design, such as metabolomic datasets.[21,22] ASCA uses an ANOVA model to decompose the data matrix into main effect and interaction matrices that contain the level averages for the experimental factors and a matrix of residuals that are not explained by the model.[23] Then, principal component analysis (PCA) is applied separately on each effect matrix to extract and represent the information in a space of reduced dimensions.[24] As ASCA is a supervised method, before interpretation of the PCA scores and loadings, the significance of factor effects and their interactions must be determined and the results examined to ensure that overfitting has not occurred. A commonly used test is the permutation test,[25] where the data variation induced by such effects is contrasted against an empirical permutation distribution obtained through resampling.[26]

Metabolic fingerprinting research shows that the chemical composition extracted from plants depends on the solvent and analytical method used.[27,28] Accurate outcomes necessitate meticulous selection of both solvent and instrumental technique.[29] Metabolic profiles, obtained from a statistical mixture design, represent a prominent approach for maximizing profile variability, thereby facilitating the comprehensive investigation of plant material.[30] These metabolic sets can be registered by different spectroscopic techniques in different spectral regions.[16,18,28,29] Employing diverse spectroscopic techniques for plant metabolomic fingerprinting analyses may yield results conducive to metabolomic investigations as they exhibit varied patterns for their spectral profiles. It is important to assess whether the extracts obtained by solvent mixture designs recorded in different spectral regions provide similar, or complementary results, in terms of the effects studied.

The main aim of this study then is to assess whether the effects of secondary sexual dimorphism (SSD, male and female) and plant growth in distinct cultivation systems (CS, monoculture and agroforestry) determined from FTIR, NIR and UV-Vis spectral profiles are significant for different solvent systems using ASCA models. Additionally, a comparison of the two factor (SSD and CS) effects obtained from these diverse spectroscopies can be made, evaluating their stabilities for varying number of repetitions (n) in the experimental factorial design, ranging from n = 3 to 5 for each level of the design.

Experimental

Leaf collection

A comprehensive description of the yerba mate experimental field, sample collection, and sample preparation can be found in other publications.[4,29] For ease of data analysis, the collected material was coded based on a two-level factorial design. The factors under investigation included secondary sexual dimorphism (coded as female (0) and male (1)) and cultivation system (coded as monoculture (0) and agroforestry (1)).

Metabolic extraction

A thorough description of the ultrasound-assisted extraction procedure is available in a previous work.[29] The solvent volumes in milliliters utilized for extraction, as *per* the statistical mixture design, are outlined in Table 1.

UV measurements

Measurements in the UV-Vis region were previously detailed in our study.[31]

Table 1. A simplex-centroid design for three components, ethanol (E), dichloromethane (D), and hexane (H), was employed. Seven experimental points were chosen to represent three pure components (E, D, and H), three binary mixtures (ED, EH, and DH), and a ternary mixture (EDH)

Design point	Volume of solvent / mL		
	Ethanol (E)	Dichloromethane (D)	Hexane (H)
E	60	0	0
D	0	60	0
H	0	0	60
ED	30	30	0
EH	30	0	30
DH	0	30	30
EDH	20	20	20

NIR measurements

The NIR absorption measurements were conducted using a DLP NIRscan Nano software (Texas Instruments Inc., Dallas, TX, USA). The spectrophotometer operated in transmittance mode and was equipped with a quartz cuvette with dimensions of 1 cm × 0.2 cm × 2.8 cm, providing an optical path of 2 mm. The NIR spectra were recorded within the wavelength range of 1700 to 900 nm, with a 3.9 nm resolution, and the measurements were performed at a temperature of 22 ± 1 °C. Each mixture design fingerprint spectrum was obtained by averaging 99 scans. For sample preparation, 1 mL of extract was used directly after extraction without any dilution. The resulting NIR spectra were saved in absorbance units and stored in '.txt' file format, with each sample containing 228 data points. Before performing ASCA modeling, the spectral set was normalized with multiplicative signal correction (MSC) and a 2nd derivative transformation calculated by the Savitzky-Golay method, using 11-point windows and a second-degree polynomial (Figure S1, Supplementary Information section). Finally, the spectra were mean-centered.

FTIR measurements

The FTIR spectral analysis of the extracts was conducted using an Agilent Cary 630 FTIR spectrometer (Santa Clara, CA, USA) equipped with an attenuated total reflectance (ATR) sampling module. The measurements were performed in transmittance mode, covering the 4000-400 cm^{-1} range with an approximate 1 cm^{-1} resolution. The analyses were carried out at 22 ºC temperature. For the spectroscopic analysis, solid extracts were used after drying. The FTIR spectra were saved in '.txt' file format, with each sample containing 3,864 data points (Figure S1).

To prepare the dataset for chemometric analysis, the spectral data were converted to absorbance units and MSC normalization was performed to correct for multiplicative effects, such as scattering and path length variations. The first derivative was calculated from the spectra to enhance spectral features, and reduce baseline shifts. Additionally, Savitzky-Golay smoothing was applied with a 7-point window to reduce noise and improve the signal-to-noise ratio. Finally, the normalized spectra were mean-centered.

ASCA modeling and analyses

In the individual ASCA modeling of yerba mate leaf metabolomic fingerprints, the data matrices had different sizes based on the number of repetitions for each mixture design. Each row in the matrix represented a different coded extract according to the factorial design, and each of the N columns corresponded to the detector systems described in UV, NIR, and FTIR sections. The matrix sizes were as following: 12 × N for 3 repetitions, 16 × N for 4 repetitions, and 20 × N for 5 repetitions.

For the simultaneous ASCA modeling with all the mixture design solvents, the yerba mate data matrix was reorganized to accommodate the augmented fingerprints. The columns (N) still corresponded to the different detector systems described in "UV, NIR, and FTIR measurements" sub-sections. Each row in this matrix represented a spectrum of a different extract coded according to the factorial design, with augmented columns by mixture design fingerprints. The matrix sizes were: 84 × N for 3 repetitions, 112 × N for 4 repetitions, and 140 × N for 5 repetitions.

Statistical 95% confidence intervals were determined by resampling tests performing 10,000 permutations, thus considering effects with *p*-values less than 0.05 as significant. More information about ASCA modeling can be found in various articles that detail the mathematical method.[21,22]

All computations for the ASCA modeling were performed using Matlab 2016 software[32] (R2016b, Natick, MA, USA) with tools from PLS_Toolbox 8.7.1[33] by Eigenvector Research (Manson, WA, USA).

Results and Discussion

The ASCA models were built, separately, from spectral fingerprinting of yerba mate extracts, obtained from different solvents, to investigate the effects of two cultivation systems (CS) on leaves from male and female plants (SSD), as well as the number of repetitions necessary for the metabolic system. The FTIR fingerprints were assessed initially, focusing on the significance of SSD that

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was determined from the dichloromethane extract only with 5 repetitions (Table 2). The variance of the SSD effect was 11.52% under the spectral profile. In the case of the ED extract, sexual dimorphism was found to be significant with an explained variance of 20.95, 22.02, and 13.13% for 3, 4, and 5 repetitions, respectively (Table 2). For the dichloromethane-hexane (DH), sexual dimorphism was determined to be significant only with 5 repetitions. The requirement for a higher number of repetitions suggested that the significance of sexual dimorphism in these extracts was dependent on the increased degrees of freedom of the ASCA model.[34] In other words, as the sample size (n) increases, the number of residual degrees of freedom also increases, tending towards greater statistical significance.[35] With more samples, the variability in the data is often more precisely assessed, and the sample means or other statistical estimates tend to converge more closely to the population parameters.[36] This increased precision can lead to smaller confidence intervals, narrower distributions, and higher statistical significance.[36] No significant sexual dimorphism was observed in the FTIR fingerprints of the ethanol (E), hexane (H), ethanol-hexane (EH) and ethanol-dichloromethane-hexane (EDH) extracts (Table 2). It was expected that the binary and ternary combinations of the extractor solvents (pure ethanol, dichloromethane, and hexane) would not detect substantial metabolic changes associated with sexual dimorphism, since only pure dichloromethane managed to reach significance with 5 repetitions. However, the presence of its significance in the ED fingerprint suggested synergic effects on the extraction process of the marker compounds.

The presence of different extractor solvent systems can indeed alter the chemical profile of the FTIR fingerprints obtained.[19] This is due to the varying solubility and affinity of different compounds for different solvents.[37] Mixtures of solvents can modify the dissolving power, polarity, viscosity, cavitation as in our case using ultrasound bath, in relation to properties of pure solvents.[37-40] Consequently, this solvation of the metabolite will undergo changes in the extraction and thus the chemical constituents extracted can differ in terms of both type and quantity.[28] This complexity in the interaction between components of the solvent and the samples can lead to non-linear changes in the spectral data.[28] As a result, the spectra from mixtures could display unique patterns that are not directly predictable from the spectra of individual components or pure solvents.[19] Understanding these intricate interactions is crucial for accurate and meaningful data analysis. It is also a reminder that the behavior of a mixture cannot always be extrapolated from the behavior of its individual components, especially in complex systems like chemical extraction.

The effect of the cultivation system was found to be significant only in the FTIR fingerprints of pure dichloromethane and DH extracts (n ≥ 4 repetitions) (Table 2). The explained variances within the range of replicates that determined the significance of the CS effect were 20.94 to 12.94% and 15.44 to 14.96%, respectively for D and DH extractors (Table 2). The E, H, ED, EH, and EDH extracts did not exhibit significance in determining the effect of cultivation system. Since the ED binary mixture was not able to determine the CS effect, probably the chemical markers of this effect, previously detected in the fingerprint of pure dichloromethane, when combined with ethanol, suffered an antagonistic effect limiting its detection.

The ASCA models of the FTIR fingerprint of the E, H, EH and EDH extractors were unable to determine any significant main effect but only found a significant interaction effect between the cultivation system and sexual dimorphism in the metabolic fingerprints of yerba mate. The interaction effect was determined for almost all fingerprints in the mid-infrared range of these different extractor systems.

FTIR fingerprinting in the dichloromethane and DH extracts of yerba mate leaves, had the ability to detect all main and interaction effects with 5 repetitions. Dichloromethane preferentially extracts compounds with moderate to low polarity, such as alkaloids, terpenes, and certain lipids.[41,42] In general, when it comes to FTIR fingerprinting, solvent blends did not lead to changes in ASCA modeling, compared to the models for individual solvents (Table 2). Pure ethanol and hexane did not reach the 95% confidence level of significance for the main effects. This is also true for the EH binary mixture. It is worth noting that ethanol is polar, dichloromethane is moderately polar, and hexane is non-polar.[42-44] Compounds with varying degrees of polarity will have different affinities for these solvents.[44] Thus, the spectral profiles characterize different metabolic sets. A noteworthy finding was the individual predictive ability of the ED extract in determining the SSD effect. Ethanol and dichloromethane have different polarities, so there can be interactions between their molecules in the mixture.[45] These interactions can affect the overall solvation properties of the mixture and might influence the solubility of certain compounds, benefiting the metabolites that characterize SSD in the FTIR fingerprint. Conversely, the ternary mixture EDH failed to determine the significance of any factor within the ASCA modeling. Mixing solvents will not always be advantageous. Once a mixture solvent offers a polarity variation,[38] for instance, the inclusion of hexane, a non-polar solvent, might decrease the overall polarity of the mixture and affect the solubility of polar compounds.[38]

Depending on the interactions, certain compounds can be more efficiently or less efficiently extracted from the mixture. It is important to emphasize that in this case, we are referring to a spectral profile that characterizes the metabolic set extracted and consequently determines the main effects and interactions by ASCA modeling.

Table 2. Variability of the secondary sexual dimorphism (SSD, male and female), cultivation system (CS, monoculture and agroforestry), and interaction (SSD × CS) effects, along with their respective *p*-values, in the ASCA model based on FTIR fingerprints of yerba mate leaves evaluated as a function of the number of experimental extractive repetitions (n = 3, 4, and 5) and the extractor system composed of ethanol (E), dichloromethane (D), and hexane (H) according to a statistical mixture design

Experimental design factors (2^2)	Number of experimental repetitions (n)		
	3	4	5
Ethanol extractor (E)			
SSD effect / %	3.66	3.97	4.37
p-value	0.6993	0.5428	0.4024
CS effect / %	6.40	6.71	5.52
p-value	0.4657	0.3480	0.3155
SSD × CS effect / %	54.22	47.61	46.92
p-value	0.0020	0.0001	0.0001
Residuals / %	35.72	41.71	43.18
Dichloromethane extractor (D)			
SSD effect / %	9.81	10.68	11.52
p-value	0.2593	0.1124	0.0467
CS effect / %	22.84	20.94	12.94
p-value	0.0527	0.0247	0.0376
SSD × CS effect / %	42.15	36.36	30.23
p-value	0.0001	0.0001	0.0001
Residuals / %	25.20	32.02	45.30
Hexane extractor (H)			
SSD effect / %	1.79	3.24	2.74
p-value	0.7692	0.5338	0.5412
CS effect / %	6.89	8.63	8.22
p-value	0.4657	0.3341	0.2467
SSD × CS effect / %	26.72	18.31	20.08
p-value	0.0560	0.0552	0.0049
Residuals / %	64.60	69.82	68.96
Ethanol/dichloromethane extractor (ED)			
SSD effect / %	20.95	22.02	13.13
p-value	0.0507	0.0102	0.0410
CS effect / %	12.75	8.88	6.52
p-value	0.1474	0.1237	0.2070
SSD × CS effect / %	16.06	14.00	12.68
p-value	0.0439	0.0158	0.0262
Residuals / %	50.24	55.10	67.67
Ethanol/hexane extractor (EH)			
SSD effect / %	9.11	5.23	6.99
p-value	0.3519	0.4419	0.2257
CS effect / %	6.65	7.28	6.78
p-value	0.4757	0.3135	0.2370
SSD × CS effect / %	18.05	26.02	26.06
p-value	0.1507	0.0164	0.0025
Residuals / %	66.19	61.47	60.17
Dichloromethane/hexane extractor (DH)			
SSD effect / %	13.50	11.66	14.54
p-value	0.1368	0.1075	0.0214
CS effect / %	16.79	15.44	14.96
p-value	0.0966	0.0477	0.0158
SSD × CS effect / %	29.65	34.31	30.47
p-value	0.0015	0.0002	0.0001
Residuals / %	40.06	38.59	40.03

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Table 2. Variability of the secondary sexual dimorphism (SSD, male and female), cultivation system (CS, monoculture and agroforestry), and interaction (SSD × CS) effects, along with their respective *p*-values, in the ASCA model based on FTIR fingerprints of yerba mate leaves evaluated as a function of the number of experimental extractive repetitions (n = 3, 4, and 5) and the extractor system composed of ethanol (E), dichloromethane (D), and hexane (H) according to a statistical mixture design (cont.)

Experimental design factors (2²)	Number of experimental repetitions (n)		
	3	4	5
	Ethanol/dichloromethane/hexane extractor (EDH)		
SSD effect / %	6.91	6.42	5.28
p-value	0.5409	0.3878	0.3540
CS effect / %	6.72	7.03	4.21
p-value	0.5450	0.3384	0.4600
SSD × CS effect / %	22.63	16.60	21.05
p-value	0.0657	0.0496	0.0102
Residuals / %	63.74	69.95	69.47
	Mixture design		
SSD effect / %	0.52	0.58	0.53
p-value	0.5888	0.4534	0.4168
CS effect / %	1.27	1.37	1.22
p-value	0.3104	0.2037	0.1741
SSD × CS effect / %	8.28	6.81	6.36
p-value	0.0040	0.0024	0.0011
Residuals / %	89.94	91.24	91.90

The results obtained from the ASCA models for the NIR metabolomic fingerprinting of yerba mate leaves using different extractor solvents (Table 3) demonstrated variations that can be compared to the results obtained from FTIR (Table 2). In the case of NIR, pure solvents were capable of extracting individual information for different effects, while binary or ternary mixtures capture the simultaneous influence of two or more effects (Table 3). NIR fingerprints obtained with ethanol showed significant determination of the cultivation system effect with explained variances ranging from 53.71 to 42.55% for 3-5 repetitions. Similarly, fingerprints obtained with dichloromethane from three replicates determine the effect of CS with a 99% confidence interval. The variances for CS observed were 70.11% for three and 51.24% for five replicates. Both ethanol and dichloromethane can interact with various types of compounds through different types of interactions, such as dipole-dipole interactions,[46] hydrogen bonding,[47] and van der Waals forces.[43] Ethanol is polar protic and can extract polar and moderately polar compounds, while dichloromethane is a polar aprotic solvent and can extract compounds with moderate to lower polarity. Thus, the NIR fingerprints of yerba mate leaves extracted with ethanol and dichloromethane can present similar profiles, characterizing the same experimental factor in ASCA modeling.

In contrast to FTIR, NIR fingerprinting with hexane demonstrated the determination of the effect of sexual dimorphism for all replicates at a 99% confidence interval (Table 3). The minimum explained variance for sexual

dimorphism was 25.12% for five replicates. Hexane is a non-polar solvent, and its interactions with compounds are primarily governed by van der Waals forces,[48] dipole-induced dipole interactions,[49] and dispersion forces.[49] This is particularly important when extracting lipids, oils, and other hydrophobic natural compounds. Anti-symmetric and symmetric stretching vibrations of methylene groups (CH_2) of metabolites with long chains of CH are characterized as FTIR markers of sexual dimorphism in yerba mate,[5] which may be characteristic of non-polar metabolites. But extraction of this marker is highly dependent on the solvent and detection method as only the ethanol-dichloromethane binary of the mixture design solvents in Table 2 results in significant SSD models. Thus, the NIR fingerprint for hexane presented a chemical profile that characterizes a different effect from that found for the extracts in ethanol and dichloromethane due to the compounds present and detected in this spectral range.

The ASCA-NIR models for the binary mixtures ED (ethanol-dichloromethane) and EH (ethanol-hexane) exhibited similar behavior in the ASCA models (Table 3). Both models successfully determined the main effects of sexual dimorphism and cultivation system from three replicates. However, they did not establish a statistically significant interaction between the two factors. As anticipated for the ASCA-NIR model for hexane, due to the inclusion of hexane in the EH mixture, it showed a higher explained variance (ranging from 75.39 to 63.15% for n = 3 to 5) for the effect of sexual dimorphism compared to ED, which had an explained variance of 31.05% for three and

30.06% for five replicates. Similarly, when examining the effect of cultivation system, the ED fingerprints exhibited more significant variances compared to EH. Specifically, ED showed significant explained variances of 24.86-27.25% (n = 3-5), while EH had values of 6.58 to 11.09% for 3 and 5 replicates, respectively (Table 3). The emergence of significance for the effect of sexual dimorphism on the NIR profile for the ED extract suggested a synergistic effect on the metabolic extraction with the binary mixture. Individually, ethanol and dichloromethane did not significantly produce this effect. The combination of these solvents in the ED binary mixture likely led to qualitative or quantitative changes in the metabolic set, resulting in the detection of sexual dimorphism. Interactions between ethanol and dichloromethane in the binary mixture can influence its solvation properties and influence the solubility of various compounds, affecting the overall chemical spectral profile,[45,47] varying the significant effects on the models.

The ASCA-NIR models for the DH (dichloromethane-hexane) and EDH (ethanol-dichloromethane-hexane) extracts of yerba mate demonstrated statistical capability in determining the main effects of secondary sexual dimorphism and cultivation system, as well as the interaction effect between these factors (Table 3). In the DH extractor system, the effect of sexual dimorphism in yerba mate was determined from three replicates, with maximum spectral variances of 32.26, 32.52, and 32.54% for 3, 4, and 5 replicates, respectively. Similarly, the effect of cultivation system was determined from three repetitions, with variances ranging from 32.37 to 32.64% for 3 to 5 repetitions. Also, the interaction effect was significant from 3 repetitions in the 99% confidence interval, with variances between 34.68 and 33.96% for 3 and 5 repetitions. Interestingly, the total spectral variance in the DH ASCA-NIR model was evenly distributed among the main effects and interaction effect, and the explained variance values showed slight variations with increasing repetitions. Furthermore, the ternary EDH mixture was capable of determining the effect of sexual dimorphism from three repetitions, with variances ranging from 25.16 to 23.66% for 3 to 5 repetitions. The effect of cultivation system was determined starting from 3 repetitions, with variances between 26.79 and 32.06% for 3 to 5 repetitions. Furthermore, the interaction effect was determined from 3 repetitions, with variances ranging from 15.81 to 20.75% for 3 to 5 repetitions. It is worth highlighting that in the case of the NIR fingerprints with the DH binary mixture, the results demonstrated a particularly favorable outcome. The DH binary mixture model displayed a low residual variance of less than 1%, indicating that it effectively explained almost all of the variance in the data and revealed the significance of all three effects for n ≥ 3.

The ASCA-FTIR five-replicate models demonstrated that both main effects could be detected simultaneously in only the dichloromethane and dichloromethane-hexane binary mixture extracts (Table 2). The ASCA-NIR models revealed that the individual extractors contained relevant information related to some of the individual effects (Table 3). Each pure extractor provided specific information about individual effects under investigation. However, the binary and ternary mixtures in the ASCA-NIR models exhibited spectral information that was capable of simultaneously determining the main effects as well as the interaction between CS × SSD. These results reinforced the understanding that NIR and FTIR techniques are not direct substitutes for each other, but rather complement each other in the analysis of samples.[50] NIR spectroscopy is particularly suitable for analyzing the overtone and combination bands of molecular vibrations.[11] It is often used for rapid, non-destructive analysis of samples, providing information about functional groups, chemical composition, and physical properties.[11,12] On the other hand, FTIR spectroscopy focuses on the fundamental vibrational bands of molecules, providing detailed information about molecular structures, bond types, and functional groups.[9,10] The different behaviors and results observed in the ASCA models for NIR and FTIR fingerprints of yerba mate leaf metabolites demonstrated that these techniques capture different aspects of the sample's chemistry and can reveal unique patterns and effects. This recognition of the different capabilities and complementary nature of FTIR and NIR further emphasizes the importance of selecting the appropriate spectroscopic technique based on the specific research objectives and the desired information about the sample under investigation.

In the models determined for the ultraviolet and visible regions (UV-Vis), Table 4, the ASCA responses differed in parts from those of FTIR (Table 2) and NIR (Table 3). The ethanol-based ASCA-UV-Vis model revealed that the interaction effect was particularly significant, starting from 3 replicates, with variances ranging from 66.57% for models with 3 replicates to 54.06% for models with 5 replicates (Table 4). ASCA-UV-Vis with E permitted the detection of significant cultivation system effect, with a variance of 22.17% for 5 replicates, while the effect of sexual dimorphism was not significant. Similarly, in the ASCA-UV-Vis model for dichloromethane, only the effect of cultivation system was determined to be statistically significant with three or more replicates. The variances for this effect were 65.54, 68.58, and 71.68% for 3, 4 and 5 replicates, respectively. In the case of hexane, the

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ASCA-UV-Vis model determined both main effects to be statistically significant at the 99% confidence level from 3 replicates. The effect of sexual dimorphism varied from 34.24 to 16.34% for 3-5 replicates, and that of the cultivation system ranged from 44.04 to 38.68% for 3 and 5 replicates, respectively. It is indeed interesting that a nonpolar solvent

Table 3. Variability of the secondary sexual dimorphism (SSD, male and female), cultivation system (CS, monoculture and agroforestry), and interaction (SSD × CS) effects, along with their respective *p*-values, in the ASCA model based on near-infrared (NIR) fingerprints of yerba mate leaves evaluated as a function of the number of experimental extractive repetitions (n = 3, 4, and 5) and the extractor system composed of ethanol (E), dichloromethane (D), and hexane (H) according to a statistical mixture design

Experimental design factors (2^2)	Number of experimental repetitions (n)		
	3	4	5
Ethanol extractor (E)			
SSD effect / %	8.53	7.44	6.83
p-value	0.1371	0.1139	0.0739
CS effect / %	53.71	47.54	42.55
p-value	0.0001	0.0001	0.0001
SSD × CS effect / %	4.12	4.37	5.73
p-value	0.3775	0.2587	0.1110
Residuals / %	33.64	40.65	44.89
Dichloromethane extractor (D)			
SSD effect / %	1.59	4.83	6.96
p-value	0.6813	0.2127	0.0992
CS effect / %	70.11	58.96	51.24
p-value	0.0042	0.0004	0.0001
SSD × CS effect / %	1.51	2.12	4.14
p-value	0.6492	0.4175	0.1837
Residuals / %	26.79	34.09	37.66
Hexane extractor (H)			
SSD effect / %	30.00	32.80	25.12
p-value	0.0111	0.0005	0.0039
CS effect / %	2.37	2.71	2.18
p-value	0.8858	0.7218	0.6871
SSD × CS effect / %	5.14	4.46	5.42
p-value	0.5576	0.4275	0.2612
Residuals / %	62.49	60.03	67.28
Ethanol/dichloromethane extractor (ED)			
SSD effect / %	31.05	31.01	30.06
p-value	0.0164	0.0014	0.0005
CS effect / %	24.86	25.78	27.25
p-value	0.0242	0.0036	0.0002
SSD × CS effect / %	0.86	1.58	4.21
p-value	0.8929	0.6786	0.1664
Residuals / %	43.23	41.63	38.48
Ethanol/hexane extractor (EH)			
SSD effect / %	75.39	67.57	63.15
p-value	0.0001	0.0001	0.0001
CS effect / %	6.58	10.88	11.09
p-value	0.0175	0.0016	0.0004
SSD × CS effect / %	2.23	0.80	0.92
p-value	0.3198	0.7971	0.6853
Residuals / %	15.80	20.75	24.84
Dichloromethane/hexane extractor (DH)			
SSD effect / %	32.26	32.52	32.54
p-value	0.0414	0.0091	0.0021
CS effect / %	32.37	32.57	32.64
p-value	0.0335	0.0082	0.0014
SSD × CS effect / %	34.68	34.28	33.96
p-value	0.0019	0.0001	0.0001
Residuals / %	0.69	0.63	0.86

Table 3. Variability of the secondary sexual dimorphism (SSD, male and female), cultivation system (CS, monoculture and agroforestry), and interaction (SSD × CS) effects, along with their respective *p*-values, in the ASCA model based on near-infrared (NIR) fingerprints of yerba mate leaves evaluated as a function of the number of experimental extractive repetitions (n = 3, 4, and 5) and the extractor system composed of ethanol (E), dichloromethane (D), and hexane (H) according to a statistical mixture design (cont.)

Experimental design factors (2^2)	Number of experimental repetitions (n)		
	3	4	5
	Ethanol/dichloromethane/hexane extractor (EDH)		
SSD effect / %	25.16	23.23	23.66
p-value	0.0128	0.0044	0.0012
CS effect / %	26.79	32.28	32.06
p-value	0.0195	0.0024	0.0004
SSD × CS effect / %	15.81	18.27	20.75
p-value	0.0190	0.0012	0.0002
Residuals / %	32.24	26.22	23.53
	Mixture design		
SSD effect / %	2.79	2.43	1.93
p-value	0.0635	0.0265	0.0215
CS effect / %	1.24	1.26	1.12
p-value	0.4272	0.2790	0.2291
SSD × CS effect / %	2.57	2.42	1.93
p-value	0.0788	0.0320	0.0237
Residuals / %	93.40	93.89	95.02

like hexane extracts compounds indicating both SSD and CS effects are significant whereas the relatively polar ones, ethanol and dichloromethane, only indicate different solitary significant effects. Some of the metabolites present in yerba mate leaves that can be detected using UV-Vis spectroscopy include caffeine, chlorogenic acid, theobromine, flavonoids and polyphenols, carotenoids, xanthophylls and tannins.[51] It is important to note that the UV-Vis fingerprint can vary based on factors such as pH, solvent, and concentration of metabolites.[52] Dichloromethane is often used to selectively extract caffeine and UV-Vis fingerprints tend to have a characteristic profile for this metabolite.[52] Normally, ethanol is used to extract chlorogenic acid from plant materials and consequently, the UV-Vis profile of this extractor tends to have characteristic absorption bands for this metabolic class associated with bands of other classes,[19] since ethanol has the capacity to extract most of the chemical classes present in yerba mate leaves.

In the ASCA-UV-Vis models, the response of binary mixtures was similar across different extractors (Table 4). All main effects and the interaction effect between these factors were statistically significant, with different variance within each model for each effect. For the ED (ethanol-dichloromethane) fingerprints, the effect of SSD was significant from four replicates, with a variance of 19.06%. The effect of cultivation system became significant from 3 repetitions, with a variance of 35.11% as well as the interaction effect with a variance of 36.01%. The effect of cultivation system and the interaction effect had the highest variances in this ASCA-UV-Vis model. Similarly,

in the ASCA-UV-Vis model based on the EH (ethanol-hexane) extractor system, the SSD effect was determined to be significant from 4 repetitions. The CS effect and the interaction effect were both significant from 3 repetitions. The effect of cultivation system had the highest variances, exceeding 70% at the 99% confidence level. In the case of the DH model based on the UV-Vis molecular profile, all effects were statistically significant starting from three repetitions. The effect of cultivation system consistently had the highest variances among the other factor effects, always exceeding 40%. However, the ASCA-UV-Vis modeling using the ternary system (EDH) only determined the statistical significance for the interaction effect starting from 3 repetitions. The variances for the interaction effect ranged from 78.14 to 68.60% for 3-5 repetitions.

Our findings demonstrate that the ASCA-UV-Vis models exhibit consistent responses for binary mixtures across different extractors in yerba mate fingerprints (Table 4). The significance and explained variances of the main effects and interaction effect may vary depending on the specific extractor system used. The interactions in solvent mixture extraction processes are complex and dynamic, influenced by a combination of chemical factors and most of the time the chemical properties of these extracting solutions need extensive mathematical formulations to be established.[43,45-47]

In ASCA modeling of a proton nuclear magnetic resonance (^{1}H NMR) dataset of yerba mate leaf fingerprints, the statistical significance of effects increases, while the explained variance decreases when the number

of experimental repetitions increase.[29] The NMR spectral profiles are effective in determining individual effects using pure extractors, as well as simultaneous effects using binary and ternary solvent combinations.[29] The variation in significance and explained variance across effects was consistent with the results obtained in ASCA-NIR and ASCA-UV-Vis models, while ASCA-FTIR showed a different pattern. The ASCA-NMR model, with 3

Table 4. Variability of the secondary sexual dimorphism (SSD, male and female), cultivation system (CS, monoculture and agroforestry), and interaction (SSD × CS) effects, along with their respective *p*-values, in the ASCA model based on ultraviolet-visible (UV-Vis) fingerprints of yerba mate leaves evaluated as a function of the number of experimental extractive repetitions (n = 3, 4, and 5) and the extractor system composed of ethanol (E), dichloromethane (D), and hexane (H) according to a statistical mixture design

Experimental design factors (2²)	Number of experimental repetitions (n)		
	3	4	5
Ethanol extractor (E)			
SSD effect / %	2.81	4.91	1.81
p-value	0.5816	0.3705	0.5392
CS effect / %	18.12	16.55	22.17
p-value	0.1821	0.1223	0.0353
SSD × CS effect / %	66.57	64.70	54.06
p-value	0.0003	0.0001	0.0001
Residuals / %	12.50	13.84	21.96
Dichloromethane extractor (D)			
SSD effect / %	3.44	2.95	2.83
p-value	0.3469	0.2572	0.1600
CS effect / %	65.54	68.58	71.68
p-value	0.0023	0.0001	0.0001
SSD × CS effect / %	9.00	6.85	3.63
p-value	0.0774	0.0523	0.0879
Residuals / %	22.02	21.62	21.86
Hexane extractor (H)			
SSD effect / %	34.24	21.41	16.34
p-value	0.0010	0.0109	0.0119
CS effect / %	44.04	40.33	38.68
p-value	0.0001	0.0001	0.0001
SSD × CS effect / %	3.32	2.32	2.10
p-value	0.2542	0.4403	0.4173
Residuals / %	18.40	35.94	42.88
Ethanol/dichloromethane extractor (ED)			
SSD effect / %	22.44	19.06	13.23
p-value	0.0614	0.0379	0.0444
CS effect / %	35.11	34.46	36.43
p-value	0.0222	0.0090	0.0023
SSD × CS effect / %	36.01	40.14	39.89
p-value	0.0014	0.0001	0.0001
Residuals / %	6.44	6.34	10.45
Ethanol/hexane extractor (EH)			
SSD effect / %	7.66	10.17	9.52
p-value	0.0741	0.0298	0.0138
CS effect / %	74.06	60.98	64.33
p-value	0.0013	0.0001	0.0001
SSD × CS effect / %	12.77	13.51	13.47
p-value	0.0004	0.0020	0.0001
Residuals / %	5.51	15.34	12.68
Dichloromethane/hexane extractor (DH)			
SSD effect / %	23.17	17.63	16.67
p-value	0.0136	0.0047	0.0026
CS effect / %	48.31	52.07	51.32
p-value	0.0011	0.0001	0.0001
SSD × CS effect / %	21.97	15.53	14.97
p-value	0.0007	0.0014	0.0001
Residuals / %	6.55	14.77	17.04

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Table 4. Variability of the secondary sexual dimorphism (SSD, male and female), cultivation system (CS, monoculture and agroforestry), and interaction (SSD × CS) effects, along with their respective *p*-values, in the ASCA model based on ultraviolet-visible (UV-Vis) fingerprints of yerba mate leaves evaluated as a function of the number of experimental extractive repetitions (n = 3, 4, and 5) and the extractor system composed of ethanol (E), dichloromethane (D), and hexane (H) according to a statistical mixture design (cont.)

Experimental design factors (2²)	Number of experimental repetitions (n)		
	3	4	5
	Ethanol/dichloromethane/hexane extractor (EDH)		
SSD effect / %	9.45	7.78	13.40
p-value	0.3291	0.2871	0.1064
CS effect / %	4.50	6.15	6.37
p-value	0.5119	0.3513	0.2511
SSD × CS effect / %	78.14	77.39	68.60
p-value	0.0011	0.0001	0.0001
Residuals / %	7.91	8.68	11.63
	Mixture design		
SSD effect / %	0.22	0.18	0.11
p-value	0.8964	0.8803	0.9142
CS effect / %	2.24	2.42	2.53
p-value	0.1409	0.0599	0.0237
SSD × CS effect / %	1.85	2.03	1.94
p-value	0.2027	0.0943	0.0585
Residuals / %	95.69	95.37	95.42

repetitions, is identified as the beginning of a statistically stable range for metabolomic data analysis,[29] while here different extractor systems were able to determine the ASCA effects simultaneously for each spectroscopic technique when 3 repetitions were used (Table 5). Simultaneously, ASCA-NIR determined the all effects and interaction in H, DH, and EDH, ASCA-UV in DH, and ASCA-NMR in ED. Regarding detection of only one significant effect, ASCA-NIR determined the effect of cultivation system on E, ASCA-UV determined the cultivation system effect on D and the interaction effect on E and EDH, and ASCA-NMR determined only the effect of sexual dimorphism on H and the interaction effect on D and EDH fingerprints. ASCA-FTIR individually determined the interaction effect on E, D, and DH fingerprints.

The row-wise augmentation approach, where the matrix is augmented by simultaneously analyzing all fingerprints from different extractor systems, is an alternative for the chemometric analysis of fingerprints in various solvent systems.[29] This method aims to maximize the variability of the chemical profile extracted from the plant by analyzing the different extractors collectively.[27] While this approach offers advantages in increasing the robustness of the method, there are also some potential disadvantages depending on the multivariate method employed.[29]

In the case of ASCA-FTIR modeling using row-wise augmentation, the results showed low percentage explained variance for the main factors and interactions, with a high percentage residual value for all replications (Table 2). Only the interaction effect was statistically significant with 3-5 spectral repetitions, while the main effects were not

Table 5. Comparison between FTIR, UV, NIR, and ¹H NMR[29] spectroscopic techniques in determining the significance of the ASCA effects for yerba mate fingerprints according to a statistical mixture design, ethanol (E), dichloromethane (D) and hexane (H) and their mixtures with 3 extracts/spectral repetitions. ASCA results according to yerba mate codification to secondary sexual dimorphism effect (SSD, male and female), cultivation system effect (CS, monoculture and agroforestry), and interaction effects (IE, SSD × CS)

Spectroscopic techniques	Extractor solvents																				
	E			D			H			ED			EH			DH			EDH		
	ASCA effects																				
	SSD	CS	IE	SSD	CS	IE	SSD	CS	IE	SSD	CS	IE	SSD	CS	IE	SSD	CS	IE	SSD	CS	IE
FTIR		×				×					×	×						×			
NIR		×			×	×	×	×	×	×	×		×	×		×	×	×	×	×	×
UV			×		×		×	×			×	×		×	×	×	×	×			×
¹H NMR	×	×			×		×			×	×	×		×	×		×	×			×

FTIR: Fourier-transform infrared spectroscopy; NIR: near-infrared spectroscopy; UV: ultraviolet; NMR: nuclear magnetic resonance.

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significant. Similarly, the results for ASCA-NIR (Table 3) and ASCA-UV-vis (Table 4) models did not demonstrate significant advantages on increasing the matrix by rows. The simultaneous ASCA-NIR model determined the significance of sexual dimorphism and the interaction effect with only a small percentage of variance, and the simultaneous ASCA-UV-Vis model only found the cultivation system effect significant. Overall, the row-wise augmentation strategy for simultaneous modeling would be more advantageous if it could determine the significance of more main factors and interactions than the individual extractors. However, in the cases of FTIR, NIR, and UV-Vis spectroscopy, there was no clear advantage associated with simultaneous spectral analysis of all extractor systems compared to the results obtained from individual extractor solvents. On the other hand, this row-wise augmentation strategy did prove effective for data obtained from ^{1}H NMR, where all factors are determined simultaneously with 5 repetitions, ensuring the significance of chemical changes across a set with wide chemical variability.[29] The chemical shift range in NMR can be quite extensive, often spanning hundreds of parts *per* million (ppm).[53]

The choice of chemometric approach and modeling strategy should be carefully considered based on the specific experimental setup and objectives of the analysis. It appears that for certain spectroscopic techniques, like ^{1}H NMR, the row-wise augmentation approach can be beneficial in capturing significant effects, while for techniques like FTIR, NIR, and UV-Vis, the advantages may be limited, and individual extractor system may be more suitable.

Conclusions

The choice of spectroscopic method for fingerprint acquisition in metabolomic analysis significantly impacted the speed, sensitivity, and accuracy of detecting substances within a plant extract. Different spectroscopic techniques, such as FTIR, NIR, UV-Vis, and NMR, exhibited variations in the fingerprint due to their unique detections of chemical systems. The use of different solvent systems for metabolic extraction also introduced substantial changes in the spectral profiles of the plant material, in addition to the diverse behaviors in ASCA modeling for the same plant extract with spectra acquired by different instrumental techniques. In this study, ASCA models were built separately from spectral fingerprints of yerba mate extracts obtained using different solvents, as well as the impact of the number of repetitions on the metabolic system. FTIR fingerprinting results showed that the interaction effect is highly pronounced in all fingerprints regardless of the

extractor system. The effect of SSD was pronounced in the ED fingerprint, while the CS effect was significant in the D and DH fingerprints. In contrast, the ASCA models for NIR fingerprints demonstrated that pure solvent systems were capable of extracting individual information for different effects, while binary and ternary mixtures could capture simultaneous influences of multiple effects. For NIR fingerprints obtained with ethanol and dichloromethane, the effect of cultivation system was significant. In the UV-Vis spectral profiles of pure systems, individual main effects were predominantly explained, except for hexane, where both main effects were determined to be significant. In the ASCA-UV-Vis models for binary mixtures, all main effects and interaction effects were statistically significant, with variations in the magnitude of variance within each model for each effect. The row-wise augmentation approach (the matrix augmented by simultaneously analyzing all fingerprints from different extractor systems), did not demonstrate significant benefits for FTIR, NIR, and UV-Vis spectroscopy. The study highlighted the importance of selecting the appropriate spectroscopic technique and solvent system based on the specific research objectives in metabolomic analysis. Different spectroscopic methods captured distinct aspects of the sample's chemistry, and their capabilities may complement each other in providing comprehensive information about the plant extracts. The findings can help on making informed choices and designing robust metabolomic future studies. The experimental metabolic extraction step outlined by the mixture design is the one that consumes the most experimental time in developing a comprehensive fingerprint. Acquiring UV-Vis, FTIR, and NIR fingerprints is a rapid, cost-effective process that does not necessitate sophisticated technical training to execute. As demonstrated, a multivariate investigation of extractive systems, as well as chemical sensing systems, can attain a global understanding of a metabolic system.

Supplementary Information

Supplementary information (graphical plotting of NIR and FTIR fingerprints) is available free of charge at http://jbcs.sbq.org.br. as PDF file.

Acknowledgments

Author Contributions

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