Prediction of fatty acids in chocolates with emphasis on C18:1 *trans* fatty acids positional isomers using ATR-FTIR associated with multivariate calibration

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ABSTRACT: The adverse effects of industrial *trans* fatty acids (TFA) consumption have prompted legislation to avoid these fats in food. This work aimed to predict C18:1 TFA isomers as well as other groups of fatty acids (saturated, monounsaturated, polyunsaturated, and total TFA) in chocolates by ATR-FTIR and partial least square regression. The quantification of fatty acids in representative samples (white, dark, and milk chocolates) was rapid (<30 s) and did not require derivatization. The optimized models showed satisfactory linear correlations when compared to a reference gas chromatographic method; coefficients of correlation for prediction ranged from 0.973 to 0.998 considering C18:1 *trans* isomers and from 0.965 to 0.999 considering fatty acids groups. The obtained results indicate that this straightforward procedure is suitable for chocolate analysis, providing its general lipid composition and TFA isomeric profile, which would be of great interest for quality control programs in the face of the new TFA regulations.

1.0 INTRODUCTION

Chocolate is a product widely consumed worldwide, and its market has grown exceptionally in recent years. Besides its flavor characteristics, some evidences suggest that chocolate intake may be associated with favorable health outcomes mainly related with a reduced risk of coronary heart disease and stroke, due to the high amounts of antioxidants present in cocoa ^{1,2}. However, the presence of other ingredients, such as fat or sugar, could be of concern. In the last decades, the massive replacements of cocoa butter in chocolates by partially hydrogenated vegetable oils (PHVO) has led to a considerable concentration of *trans* fatty acids (TFA) in chocolate products ³⁻⁵, which may exert deleterious health effects on human health related to an increased risk of cardiovascular disease ⁶.

The awareness of the adverse effects associated with the consumption of TFA from industrial origin has prompted regulatory agencies to enforce legislation aiming PHVO reduction or prohibition in food. Strict laws in the United States and Canada prohibited the use of this fat in food products ^{7,8}, while in Europe, the presence of TFA from industrial origin in food has been recently restricted to a maximum of 2% per total fat ⁹. It is important to emphasize that TFA from

natural origin, present in meat and dairy products, are not included in these limits since these fats would not cause the same adverse effects in human health ^{10,11}. Denmark was the pioneer country that introduced legal limits to industrial *trans* fats in 2004, reducing mortality related to cardiovascular disease significantly in its population ¹². Nowadays, many countries such as Brazil, Paraguay, Uruguay, Argentina, or South Korea have no restriction limits for PHVO addition in foods, but the total amount of TFA should be informed in the nutrition facts label ¹³. Therefore, the development of fast, sensitive, and accurate methods able to quantify total TFA content and discriminate natural from industrial *trans* fats is urgently needed.

In chocolate products, TFA origin can be both from industrial and natural sources, since dairy fat and PHVO may be used in chocolate manufacture ¹⁴. However, a maximum of 5% of cocoa butter replacement is regulated in several countries ^{15,16}. In the United States, chocolate shall not contain any fat other than cocoa butter or dairy fat in its composition ¹⁷. Official Gas Chromatography (GC) ^{18,19} and Fourier Transform Infrared (FTIR) ^{20,21} methods are generally used for TFA determination in foods. A complete fatty acid (FA) profile can be determined by GC, including TFA isomers. However, GC is not adequate for routine analysis,

since long analysis time is required. Besides, derivatization reactions are mandatory to convert FA into more volatile compounds, such as fatty acid methyl esters (FAME) or homologues, and this procedure may result in sample preparation errors²². On the other hand, the FTIR official methodology provides a faster analysis time, but only total TFA content is determined.

The potential of FTIR for chocolate quality control has already been demonstrated for the antioxidant capacity prediction^{23,24} and to detect potential adulteration with lard²⁵. Nevertheless, to the best of our knowledge, a quantitative approach based on FTIR to determine TFA profiling in chocolate samples has never been reported. Besides, although the potential of FTIR has already been demonstrated for TFA prediction in samples containing TFA from natural origin (milk)²⁶ and industrial origin (oils and fats)²⁷, this is the first time that a matrix containing TFA from both origins is being approached.

This work aimed to investigate the prediction 18:1 TFA isomers (*trans* 6-8, *trans* 9, *trans* 10, *trans* 11 and *trans* 12), the most abundant *trans* fats in food products, in chocolates, and also its general lipid composition (total TFA, saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA)) by attenuated total reflectance Fourier Transform Infrared (ATR-FTIR) associated with multivariate calibration. This approach is limited for C18:1 isomers since the TFA in PHVO are generated during hydrogenation of vegetable oils, which are a source of PUFA with the same chain length (C18:2 and C18:3), generating C18:1 TFA²⁸. Also, C18:1 TFA are major *trans* isomers in milk¹¹. This straightforward and rapid methodology has potential to be an alternative to the official methods for predicting the TFA isomeric content and the general lipid composition of chocolates. It would also be relevant for food industries to perform quality control analysis and also for regulatory agencies in order to investigate food fraud.

2. MATERIALS AND METHODS

2.1. Samples and Reagents. Thirty-two chocolate samples (white, dark, and milk chocolates) from different national and multinational brands produced in Brazil were used to carry out this research. Samples were purchased in local markets. The addition of PHVO is still acceptable in this country, and some products had high *trans* fats contents declared. All reagents used in the experiments were of analytical grade, and water used in aqueous solutions was purified by deionization (Milli-Q system; Millipore, Bedford, MA, USA). Methanol, potassium hydroxide (KOH) and sodium bisulfate (NaHSO₄) were purchased from Panreac Química S.A. (Madrid, Spain). Hexane, isopropanol, and sodium sulfate (Na₂SO₄) were obtained from Vetec (Rio de Janeiro, RJ, Brazil). Analytical FAME standards (GLC-603, GLC-611, GLC-643, GLC-80, GLC-409) were acquired from Nu-Chek Prep Inc. (Elysian, MN, USA).

2.2 Lipid Extraction Procedure. Lipid extraction was performed according to Hara and Radin adapted procedure²⁹. 2 g of sample were weighed in a flask; 7.5 mL of extraction solution (hexane and isopropanol 3:2 v/v) and 5 mL of an aqueous Na₂SO₄ solution (0.5 mol L⁻¹) were added. Then, the solution was mixed in a vortex for 1 min, sonicated for 15 min, and centrifuged for 5 min (10,000 rpm). The organic

layer (upper) was transferred to another flask, and the aqueous layer (lower) was washed twice with 5 mL of extraction solution. The pooled organic extract was evaporated in a rotary evaporator at 40 °C, resuspended in 500 µL of hexane, and transferred to an amber vial, where chocolate fat was stored under refrigeration in an inert atmosphere.

2.3. Gas Chromatography Analysis. Sample preparation and analysis by gas chromatography was performed as reported previously³⁰. 25 mg of chocolate fat were weighed in a microtube, and 200 µL of hexane were added. The solution was vortexed, and 50 µL of a methanolic KOH solution (2 mol L⁻¹) were added to the microtube, vortexed again for 1 min. After 5 min rest, 125 mg of NaHSO₄ were added to the microtube, which was centrifuged for 5 min at 0°C (10,000 rpm). Then, the solution was transferred to GC vials and diluted in a proportion of 1:10 v/v with hexane.

A GC 6890 N from Agilent (Palo Alto, CA, USA) equipped with a flame ionization detector (FID), auto-injector, and a CP-SIL capillary column (100 m, 0.25 mm x 0.2µm, Varian, Middelburg, The Netherlands) was used for FAME quantification. FID detector and injector temperatures were fixed at 250 °C, and split ratio was 1:100. The GC temperature program employed was: the initial temperature at 45 °C; after 4 min, the temperature was raised at 13 °C min⁻¹ to 165 °C and hold for 35 min, then increased to 215 °C at 4 °C min⁻¹ and kept for 30 min.

Another GC 6890 A from Agilent (Palo Alto, CA, USA) equipped with a single quadrupole electron impact mass spectrometer detector (MS), autosampler, and a column with the same stationary phase and dimensions was also used for FAME identification. Ion source and interface MS temperatures were 150 and 230°C, respectively. Wiley 275 and NIST 05 libraries were used to identify the mass spectra.

FAME identification and quantification were also carried out as reported previously³⁰. FAME were firstly identified by comparison of the elution order with analytical standards from Nu-Chek Prep Inc., and previous reports²⁸. FAME identities were confirmed by GC-MS. A butter oil reference standard (CRM 164; European Community Bureau of Reference, Brussels, Belgium) with certified composition was used for the quantification of samples by GC-FID. Response factors were calculated as recommended by ISO International Standard³¹.

2.4 ATR-FTIR spectra acquisition. FTIR spectra of chocolate fat were recorded using the Perkin-Elmer® Spectrum™ 400 FT-IR/NIR spectrometer (Perkin Elmer Inc., Madrid, Spain) in middle infrared mode and equipped with an ATR sampling device. Spectra were acquired considering the wavenumber range of 4000-650 cm⁻¹, with 16 scans at 4 cm⁻¹ resolution. Following each spectral acquisition, the ATR accessory was cleaned with tissue paper and isopropanol. A background single-beam spectrum was collected from was collected from a black optical path after eight measurements.

2.5 Multivariate data analysis. Multivariate data analysis was performed by using PLS Toolbox chemometrics software (PLS_Toolbox_4.1, Eigenvector Research Inc., Wenatchee, WA) that runs in a MATLAB computational environment (MATLAB R2019a, The MathWorks Inc., Natick,

MA, USA). Partial least square regression (PLSR) was used to establish quantitative models between the spectral matrix X and quantitative matrix Y, containing FA contents obtained by GC-FID (expressed as g/ 100 g of total FA). Models were optimized for 18:1 TFA isomers (*trans* 6-8, *trans* 9, *trans* 10, *trans* 11 and *trans* 12) and FA groups (Σ TFA, Σ SFA, Σ MUFA, Σ PUFA). Data were evaluated without any spectral preprocessing, and employing two commonly used preprocessing procedures: standard normal variate (SNV)³² and the Savitzky-Golay smoothing algorithm³³. Besides evaluating the use of full spectra for modeling construction, two procedures were assessed for variable selection: interval PLS (iPLS - partitioning spectral data in 20 intervals and considering one interval at a time) and synergic interval PLS (siPLS - partitioning spectral data in 20 intervals and considering the combination of two intervals at a time). 70% of chocolate samples were used for model calibration, employing cross-validation, and 30% of chocolate samples were employed for the external validation (prediction). Samples used in the prediction step were selected by organizing data in crescent content order and selecting one within three samples. PLSR models were evaluated considering the coefficient of correlation for cross-validation (r_{cv}), coefficient of correlation for prediction (r_p), root mean square error for cross-validation (RMSECV), and root mean square error for prediction (RMSEP). Ten latent variables (LV) were investigated in each model.

3. RESULTS AND DISCUSSION

3.1 ATR-FTIR spectra of Chocolate samples. Fig. 1 shows the full spectra of all chocolates analyzed, used to PLS modeling, also indicating the characteristic bands of FA, and the 20 intervals appraised (from 640 to 4000 cm^{-1} with increments of 167.5 cm^{-1}) when evaluating iPLS and siPLS algorithms. This step of variable selection is critical in the multivariate calibration of the infrared spectra, and it can

improve the prediction performance of the model, providing a more straightforward interpretation of data³⁴, which justifies its evaluation in the modeling.

In Fig 1, a broad band, centered at approximately 3340 cm^{-1} , can be assigned to the overtone of the glyceride ester carbonyl absorption, and a shoulder around 3006 cm^{-1} can be observed due to the stretching mode of the *cis* olefinic double bonds of FA. The CH_3 asymmetrical stretching mode causes a shoulder at 2953 cm^{-1} , and both the methylene asymmetrical stretching and the methylene symmetrical stretching modes can be observed at approximately 2924 cm^{-1} and 2853 cm^{-1} , respectively²⁵. The C=O group of triglycerides shows a stretching vibrational mode at 1746 cm^{-1} , and the small band at 1655 cm^{-1} can be attributed to C=C stretching vibration of disubstituted *cis* C=C of acyl groups of oleic and linoleic acids³⁵. Bending and rocking vibrational modes regarding CH, CH_2 , and CH_3 groups can be observed between 1465 and 1370 cm^{-1} ³⁶. The band at 1377 cm^{-1} can be assigned to the symmetrical bending vibration of methyl groups²⁵ and the signals observed at approximately 1238 cm^{-1} , 1163 cm^{-1} , 1118 cm^{-1} , and 1097 cm^{-1} and a shoulder at 1033 cm^{-1} could be assigned to the stretching vibrations of the C-O group in esters³⁵. The band observed at 966 cm^{-1} is due to the C=C-H bending vibrational mode of *trans* olefin, which is of substantial importance in this work. Official methods have reported measurements of this band area for the determination of total TFA content in samples^{20,21}. Finally, the band at 723 cm^{-1} can be assigned to the overlapping of the methylene rocking vibration and the out-of-plane bending vibrational mode of *cis*-disubstituted olefins³⁵.

Fig 2 shows the ATR-FTIR spectra of chocolates obtained after applying two commonly used preprocessing procedures: SNV (Fig 2-A) and the Savitzky-Golay smoothing algorithm (Fig 2-B). Spectral preprocessing is frequently used before multivariate data analysis to reduce variations in instrumental responses and intrinsic spectral backgrounds,

Fig. 1: ATR-FTIR raw spectra (divided into 20 intervals) of the chocolates analyzed with their characteristic bands.

resulting in a more reliable spectral data ³⁷. ATR-FTIR spectra may contain variations produced from flaws or intrinsic limitations related to the instrumentation, as low signal intensity or scattering. Thus, scattering correction algorithms, as SNV or Multiplicative Scatter Correction, are recommended before modeling. Additionally, to increase the signal-to-noise and accentuate the authentic spectral features, the Savitzky-Golay smoothing algorithm can be employed ³⁸.

3.2 Partial least square regression analysis. PLSR models were optimized for 18:1 TFA isomers (*trans* 6-8, *trans* 9, *trans* 10, *trans* 11 and *trans* 12) and four FA classes: Σ TFA, Σ SFA, Σ MUFA, Σ PUFA. Table 1 shows all models evaluated for TFA isomers, and Table 2 shows the results obtained for the FA classes. Table 3 shows the concentration range of chocolate samples used in calibration and prediction steps of multivariate calibration, calculated by the GC-FID reference method. The best models were selected considering the smallest RMSECV and RMSEP, as well as r_{cv} and r_p closest to 1. Optimum models are shown in bold in Tables 1 and 2. Based on the optimum models, correlation graphics were carried out to visually observe the agreement between the reference values obtained by GC-FID and the predicted values from ATR-FTIR-PLSR (Fig 3). GC-FID and ATR-FTIR-PLSR results used for Fig 3 construction are also shown in Tables 1 and 2 of Supporting Information.

Combined regions of the ATR-FTIR spectra generated the best models for the prediction of individual 18:1 TFA isomers and FA classes (Tables 1 and 2). More specifically, none of the selected models used the entire spectral region, and siPLS provided better results for all FA investigated. Furthermore, the 818-986 cm^{-1} region, which includes the characteristic band of *trans* C=C-H bending vibration at 966 cm^{-1} , was covered in all models selected for TFA. Regarding the preprocessing data, the Savitzky-Golay smoothing algorithm was found to be effective in correcting minor baseline shifts in the ATR-FTIR spectra and thus, yielding better performance models for 18:1 *trans* 10, 18:1 *trans* 11, and SFA.

SNV algorithm was selected for PUFA modeling. Other TFA isomers and FA classes were modeled without any preprocessing. Moreover, the low numbers of latent variables indicate that well fitted and parsimonious models were achieved ($LV < 6$ for TFA isomers and < 7 for FA classes).

The RMSE is a frequently used parameter to measure the differences between predicted values and calculated values obtained from a reference method. It indicates the accuracy of the calibration and prediction performance. The low RMSECV and RMSEP values calculated showed that all models had a good predictive accuracy: for individual 18:1 TFA isomers, RMSECV varied from 0.04 to 0.07 g/100 g of total FA and RMSEP from 0.04 to 0.10 g/100 g of total FA (Table 1); for FA classes RMSECV ranged from 0.23 to 1.87 g/100 g of total FA and RMSEP from 0.18 to 2.98 g/100 g of total FA (Table 2).

According to Shenk & Westerhaus (1996)³⁹, beyond using RMSE to estimate the accuracy of a calibration equation, it is necessary to evaluate the coefficient of determination (r^2). An r^2 value greater than 0.90 indicates excellent quantitative information, while an r^2 value between 0.50 and 0.89 indicates good quantitative information. The coefficients of correlation calculated in all optimized models show the good linearity of the models, which can also be observed in Fig 3. In the cross-validation step, $r_{cv} > 0.986$ were calculated for individual 18:1 TFA isomers models (Table 1) and $r_{cv} > 0.985$ for the FA classes (Table 2). In the prediction step, r_p varied from 0.973 to 0.998 for individual 18:1 TFA isomers and from 0.965 to 0.999 for FA classes. Thus, all optimized models resulted in $r^2 > 0.90$. These excellent correlations suggest that the developed models may be applied to a wide variety of chocolates to estimate the 18:1 TFA isomeric profile and total contents of TFA, SFA, MUFA and

Fig. 2: ATR-FTIR spectra of the chocolates analyzed after applying two pre-processing procedures: (A) standard normal variate and (B) the Savitzky-Golay smoothing algorithm.

Table 1. Models evaluated for 18:1 trans fatty acids (FA) isomers (6-8, 9, 10, 11, and 12). Optimum selected models are presented in bold.

FA	Model	Preprocessing	Spectral range (cm ⁻¹)	LVs	RMSECV*	RMSEP*	r _{cv}	r _p
Σ C18:1 trans 6-8	PLS	no	4000-650	8	0.25	0.09	0.704	0.957
	iPLS	no	986-818	4	0.08	0.07	0.970	0.967
	siPLS	no	2330-2162 & 986-818	5	0.06	0.07	0.986	0.973
	PLS	SNV	4000-650	7	0.23	0.13	0.620	0.909
	iPLS	SNV	986-818	5	0.09	0.06	0.971	0.980
	siPLS	SNV	2330-2162 & 986-818	5	0.07	0.07	0.973	0.967
	PLS	Savitzky-Golay	4000-650	8	0.20	0.10	0.726	0.945
	iPLS	Savitzky-Golay	986-818	4	0.07	0.05	0.974	0.983
	siPLS	Savitzky-Golay	3667-3500 & 986-818	3	0.06	0.05	0.978	0.984
C18:1 trans 9	PLS	no	4000-650	3	0.38	0.19	0.489	0.887
	iPLS	no	986-818	4	0.07	0.07	0.985	0.984
	siPLS	no	1994-1826 & 986-818	5	0.06	0.07	0.987	0.986
	PLS	SNV	4000-650	7	0.34	0.16	0.563	0.928
	iPLS	SNV	986-818	7	0.11	0.06	0.957	0.994
	siPLS	SNV	3166-2999 & 986-818	7	0.08	0.07	0.978	0.986
	PLS	Savitzky-Golay	4000-650	8	0.30	0.1	0.684	0.970
	iPLS	Savitzky-Golay	986-818	5	0.07	0.06	0.985	0.994
	siPLS	Savitzky-Golay	3166-2999 & 986-818	5	0.04	0.07	0.997	0.991
C18:1 trans 10	PLS	no	4000-650	7	0.34	0.16	0.777	0.974
	iPLS	no	986-818	3	0.13	0.10	0.967	0.984
	siPLS	no	1658-1490 & 986-818	4	0.10	0.11	0.982	0.981
	PLS	SNV	4000-650	6	0.33	0.18	0.734	0.958
	iPLS	SNV	986-818	6	0.11	0.08	0.977	0.993
	siPLS	SNV	2330-2162 & 986-818	5	0.08	0.11	0.990	0.982
	PLS	Savitzky-Golay	4000-650	8	0.25	0.10	0.856	0.984
	iPLS	Savitzky-Golay	986-818	3	0.10	0.08	0.984	0.994
	siPLS	Savitzky-Golay	1490-1322 & 986-818	5	0.07	0.08	0.994	0.991
C18:1 trans 11	PLS	no	4000-650	7	0.39	0.16	0.773	0.978
	iPLS	no	986-818	4	0.13	0.12	0.970	0.986
	siPLS	no	1490-1322 & 986-818	5	0.07	0.10	0.992	0.988
	PLS	SNV	4000-650	7	0.34	0.16	0.761	0.973
	iPLS	SNV	986-818	5	0.10	0.14	0.985	0.975
	siPLS	SNV	3333-3166 & 986-818	5	0.08	0.12	0.989	0.983
	PLS	Savitzky-Golay	4000-650	8	0.29	0.14	0.854	0.975
	iPLS	Savitzky-Golay	986-818	3	0.11	0.13	0.980	0.982
	siPLS	Savitzky-Golay	1490-1322 & 986-818	5	0.08	0.11	0.990	0.986
C18:1 trans 12	PLS	no	4000-650	3	0.33	0.19	0.571	0.897
	iPLS	no	986-818	4	0.06	0.05	0.991	0.993
	siPLS	no	2665-2498 & 986-818	6	0.04	0.04	0.996	0.998
	PLS	SNV	4000-650	8	0.28	0.09	0.662	0.977
	iPLS	SNV	986-818	6	0.05	0.04	0.994	0.998
	siPLS	SNV	3667-3500 & 986-818	6	0.05	0.06	0.992	0.991
	PLS	Savitzky-Golay	4000-650	8	0.25	0.10	0.763	0.971
	iPLS	Savitzky-Golay	986-818	5	0.03	0.04	0.997	0.997
	siPLS	Savitzky-Golay	2832-2665 & 986-818	5	0.03	0.05	0.997	0.996

*Expressed as g/100 g total FA; Coefficient of correlation for cross-validation (r_{cv}), coefficient of correlation for prediction (r_p), fatty acids (FA), interval partial least square (iPLS), latent variables (LV), partial least square (PLS), root mean square error for cross-validation (RMSECV), root mean square error for prediction (RMSEP), standard normal variate (SNV), synergic interval partial least square (siPLS).

Table 2. Models evaluated for fatty acids (FA) groups: saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), and total trans fatty acids (TFA). Optimum selected models are presented in bold.

FA	Model	Preprocessing	Spectral range (cm ⁻¹)	LVs	RMSECV*	RMSEP*	r _{cv}	r _p
Σ SFA	PLS	no	4000-650	8	5.07	2.07	0.939	0.995
	iPLS	no	1490-1322	4	1.41	1.89	0.996	0.996
	siPLS	no	2330-2162 & 1490-1322	6	1.13	1.96	0.997	0.996
	PLS	SNV	4000-650	8	5.19	2.19	0.938	0.995
	iPLS	SNV	1490-1322	4	1.81	2.06	0.994	0.994
	siPLS	SNV	2330-2162 & 1490-1322	4	1.44	2.39	0.995	0.992
	PLS	Savitzky-Golay	4000-650	8	1.65	1.74	0.996	0.996
	iPLS	Savitzky-Golay	1490-1322	5	1.18	1.26	0.997	0.998
	siPLS	Savitzky-Golay	2832-2665 & 1490-1322	7	1.03	1.10	0.998	0.999
Σ TFA	PLS	no	4000-650	7	1.72	0.60	0.726	0.979
	iPLS	no	986-818	4	0.30	0.22	0.992	0.997
	siPLS	no	2832-2665 & 986-818	7	0.23	0.18	0.995	0.998
	PLS	SNV	4000-650	7	1.48	0.71	0.689	0.957
	iPLS	SNV	986-818	7	0.22	0.21	0.994	0.998
	siPLS	SNV	3667-3500 & 986-818	6	0.19	0.30	0.997	0.992
	PLS	Savitzky-Golay	4000-650	8	1.27	0.44	0.794	0.982
	iPLS	Savitzky-Golay	986-818	4	0.19	0.23	0.996	0.997
	siPLS	Savitzky-Golay	3166-2999 & 986-818	4	0.16	0.26	0.997	0.996
Σ MUFA	PLS	no	4000-650	2	4.23	3.98	0.895	0.947
	iPLS	no	2999-2832	4	3.01	3.66	0.948	0.941
	siPLS	no	2999-2832 & 1490-1322	4	1.87	2.98	0.985	0.965
	PLS	SNV	4000-650	2	4.41	3.89	0.884	0.942
	iPLS	SNV	2999-2832	4	2.98	3.84	0.950	0.944
	siPLS	SNV	4000-3834 & 2999-2832	5	1.83	3.84	0.982	0.931
	PLS	Savitzky-Golay	4000-650	8	2.72	2.79	0.957	0.971
	iPLS	Savitzky-Golay	1154-986	2	2.75	2.87	0.960	0.967
	siPLS	Savitzky-Golay	2498-2330 & 1490-1322	7	1.68	3.71	0.983	0.941
Σ PUFA	PLS	no	4000-650	8	3.24	0.95	0.902	0.995
	iPLS	no	1490-1322	8	1.34	0.87	0.982	0.996
	siPLS	no	3166-2999 & 1490-1322	5	1.41	1.41	0.983	0.987
	PLS	SNV	4000-650	8	1.90	0.94	0.963	0.995
	iPLS	SNV	3166-2999	6	0.81	0.48	0.993	0.999
	siPLS	SNV	3166-2999 & 1490-1322	7	0.39	0.60	0.998	0.998
	PLS	Savitzky-Golay	4000-650	8	2.07	1.27	0.965	0.991
	iPLS	Savitzky-Golay	3166-2999	4	1.46	1.46	0.985	0.986
	siPLS	Savitzky-Golay	3166-2999 & 1490-1322	6	0.50	0.61	0.997	0.998

*Expressed as g/100 g total FA; Coefficient of correlation for cross-validation (r_{cv}), coefficient of correlation for prediction (r_p), fatty acids (FA), interval partial least square (iPLS), latent variables (LV), partial least square (PLS), root mean square error for cross-validation (RMSECV), root mean square error for prediction (RMSEP), standard normal variate (SNV), synergic interval partial least square (siPLS).

Table 3: Concentration range of chocolate samples used to calibration and prediction steps of multivariate calibration, calculated by gas chromatography (GC) reference method.

Trans fatty acids and fatty acid classes	GC	
	Calibration	Prediction
<i>trans</i> (6-8)	0.02-1.06	0.02-0.99
<i>trans</i> -9	0.03-1.49	0.03-1.28
<i>trans</i> -10	0.04-1.89	0.04-1.93
<i>trans</i> -11	0.10-2.25	0.06-2.27
<i>trans</i> -12	0.03-1.32	0.02-1.39
Σ TFA	0.31-7.53	0.23-7.90
Σ SFA	19.55-96.01	19.03-97.16
Σ MUFA	3.21-39.62	2.23-40.87
Σ PUFA	0.49-32.11	0.47-32.69

Trans fatty acid (TFA), saturated fatty acid (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA).

All values are expressed in g/100 g total fatty acids.

Fig. 3: Correlation between gas chromatography (reference method) and ATR-FTIR associated with partial least square regression (PLSR) results considering the best models obtained for the fatty acids prediction. Graph A shows calibration and prediction results for saturated fatty acids (SFA), mono-unsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA) and total *trans* fatty acids (TFA); Graph B considers the individual TFA isomers: *trans* 6-8, *trans* 9, *trans* 10, *trans* 11, and *trans* 12.

PUFA, especially aiming fraud detection and mislabeling of chocolate products.

Furthermore, it is important to remark that although the prediction models presented a small limitation for some isolated values mainly related to concentrations very close to 0 (Tables 1 and 2, Supporting Information), no significant difference was evidenced between GC and ATR-FTIR-PLSR results within the 95% confidence interval. Paired t-tests were carried out considering samples from the prediction step, and p-values > 0.05 were observed for all TFA isomers and FA classes. Parametric t-tests were performed for all FA, except for C18:1 *trans* 11, whose assumption of normality was not fulfilled (p-value = 0.022). Then, the non-parametric Wilcoxon test was carried out for this specific TFA.

In conclusion, ATR-FTIR spectroscopy associated with PLSR was useful to determine individual 18:1 TFA isomers and the general lipid composition (Σ TFA, Σ SFA, Σ MUFA, Σ PUFA) of chocolate samples. It provided excellent estimations well correlated with the reference values supplied by GC-FID. Besides, no significant difference was evidenced between GC and ATR-FTIR results within the 95% confidence interval considering paired tests. The main advantages of this methodology are that it requires a small amount of sample (approximately 10 μ L of fat), minimum sample preparation, and each spectrum can be obtained in less than 30 s, in contrast to long traditional chromatographic methods. This simple procedure would be very useful and convenient for fraud detection and mislabeling evaluation of chocolates.

ASSOCIATED CONTENT

Supporting Information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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ABBREVIATIONS

ATR-FTIR, attenuated total reflectance Fourier Transform Infrared; FA, fatty acid; FAME, fatty acid methyl esters; FID, flame ionization detector; FTIR, Fourier Transform Infrared; GC, Gas Chromatography; iPLS, interval partial least

square, LV, latent variables; MS, mass spectrometry; MUFA, monounsaturated fatty acids; PHVO, partially hydrogenated vegetable oil; PLS, partial least square; PUFA, polyunsaturated fatty acids; Rcv, Coefficient of correlation for cross-validation; RMSECV, root mean square error for cross-validation; RMSEP, root mean square error for prediction (RMSEP); Rp, coefficient of correlation for prediction; SFA, saturated fatty acids; siPLS, synergic interval partial least square; SNV, standard normal variate; TFA, trans fatty acids.

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Table 1 Supporting Information. GC-FID and ATR-FTIR data considering calibration and prediction steps of multivariate calibration for fatty acids groups.

Σ TFA				Σ SFA				Σ MUFA				Σ PUFA			
Calibration		Prediction		Calibration		Prediction		Calibration		Prediction		Calibration		Prediction	
GC-FID	ATR-FTIR	GC-FID	ATR-FTIR	GC-FID	ATR-FTIR	GC-FID	ATR-FTIR	GC-FID	ATR-FTIR	GC-FID	ATR-FTIR	GC-FID	ATR-FTIR	GC-FID	ATR-FTIR
2.15	1.94	3.87	3.65	84.81	86.17	78.52	80.52	13.59	13.11	19.39	15.57	0.73	0.99	0.92	1.86
2.38	2.32	0.35	0.54	93.2	94.84	97.16	96.99	5.74	8.38	2.23	6.74	0.58	0.74	0.47	0.20
0.55	0.63	0.41	0.41	61.31	61.02	61.14	60.87	31.13	31.36	33.04	32.68	4.96	4.76	3.44	3.52
0.38	0.90	7.9	7.57	60.19	59.01	19.03	18.88	31.16	32.64	39.12	39.19	5.88	4.91	32.69	33.27
0.36	0.51	0.35	0.40	62.51	61.72	63.22	64.19	32.17	33.84	30.55	31.77	3.06	3.30	3.79	2.59
7.53	6.82	0.46	0.42	19.55	19.48	63.93	63.58	39.31	38.42	31.08	30.99	32.11	31.78	2.68	2.79
0.39	0.44	0.3	0.28	63.98	63.97	50.89	53.24	31.12	33.23	40.87	34.70	2.74	2.83	4.93	5.98
0.40	0.4	0.23	-0.08	63.95	63.8	61.00	61.59	31.24	32.1	33.58	29.43	2.75	2.70	2.99	3.09
0.35	0.46	0.71	0.9	63.93	63.73	62.61	63.3	31.02	29.79	31.84	31.92	2.76	3.06	2.99	3.19
0.39	0.29	0.57	0.48	64.04	64.6	59.96	61.26	30.8	29.9	33.49	31.13	2.86	3.02	4.01	4.21
0.46	0.36	0.81	0.94	63.94	64.38	61.11	60.88	31.05	30.72	32.94	33.45	2.7	2.63	3.38	3.48
0.31	0.56	-	-	96.01	92.89	-	-	3.21	8.20	-	-	0.49	0.17	-	-
0.72	0.68	-	-	62.82	63.4	-	-	32.14	31.15	-	-	2.7	2.99	-	-
0.56	0.54	-	-	63.8	62.96	-	-	31.25	33.63	-	-	2.64	2.61	-	-
0.65	0.74	-	-	62.68	62.32	-	-	31.81	31.3	-	-	2.97	2.8	-	-
0.71	0.69	-	-	62.62	63.42	-	-	31.99	32.71	-	-	2.87	2.84	-	-
0.57	0.38	-	-	59.47	60.04	-	-	31.97	32.36	-	-	5.82	5.68	-	-
0.88	1.14	-	-	59.51	59.8	-	-	30.24	29.41	-	-	7.17	6.80	-	-
7.15	7.05	-	-	54.33	53.55	-	-	39.62	35.39	-	-	3.27	4.42	-	-
0.46	0.26	-	-	60.53	60.86	-	-	33.38	32.11	-	-	3.48	3.20	-	-
0.85	0.66	-	-	60.76	62.36	-	-	33.11	33.16	-	-	3.36	3.48	-	-

Trans fatty acid (TFA), saturated fatty acid (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA).

Table 2 Supporting Information. GC-FID and ATR-FTIR data considering calibration and prediction steps of multivariate calibration for trans fatty acids isomers.

C18:1 trans 6-8				C18:1 trans 9				C18:1 trans 10				C18:1 trans 11				C18:1 trans 12			
Calibration		Prediction		Calibration		Prediction		Calibration		Prediction		Calibration		Prediction		Calibration		Prediction	
GC-FID	ATR-FTIR	GC-FID	ATR-FTIR	GC-FID	ATR-FTIR	GC-FID	ATR-FTIR	GC-FID	ATR-FTIR	GC-FID	ATR-FTIR	GC-FID	ATR-FTIR	GC-FID	ATR-FTIR	GC-FID	ATR-FTIR	GC-FID	ATR-FTIR
0.19	0.26	0.36	0.47	0.45	0.40	0.77	0.69	0.5	0.44	0.95	0.77	0.66	0.58	1.15	1.13	0.34	0.36	0.65	0.66
0.28	0.19	0.09	-0.04	0.48	0.46	0.11	0.12	0.6	0.48	0.07	0.18	0.65	0.65	0.06	0.34	0.38	0.33	0.03	0.02
0.04	0.05	0.07	0.03	0.05	0.07	0.05	0.03	0.08	0.11	0.08	0.04	0.31	0.28	0.16	0.2	0.07	0.08	0.05	0.04
0.03	-0.03	0.99	0.95	0.05	0.07	1.28	1.17	0.09	0.04	1.93	1.92	0.16	0.24	2.27	2.22	0.06	0.07	1.39	1.28
0.02	0.05	0.02	0.07	0.03	0.05	0.05	0.06	0.06	0.06	0.05	0.05	0.22	0.29	0.21	0.24	0.04	0.05	0.03	0.06
0.95	0.88	0.04	0.06	1.17	1.14	0.04	0.03	1.89	1.67	0.06	0.03	2.25	2.14	0.25	0.21	1.27	1.15	0.07	0.07
0.05	0.05	0.02	0.03	0.05	0.04	0.03	-0.07	0.05	0.03	0.04	0.04	0.19	0.31	0.19	0.25	0.06	0.04	0.02	0.02
0.04	0.07	0.04	0.02	0.05	0.04	0.06	-0.06	0.06	0.05	0.04	-0.08	0.2	0.23	0.06	-0.06	0.05	0.07	0.03	-0.01
0.04	0.07	0.04	0.08	0.04	0.07	0.07	0.13	0.04	0.04	0.09	0.15	0.19	0.14	0.42	0.42	0.03	0.08	0.09	0.12
0.05	0.04	0.08	0.07	0.05	0.01	0.09	0.05	0.05	0	0.07	0.07	0.2	0.15	0.25	0.21	0.04	0.05	0.09	0.06
0.07	0.05	0.05	0.13	0.05	0.04	0.09	0.12	0.06	0.02	0.09	0.15	0.22	0.18	0.49	0.41	0.06	0.04	0.10	0.13
0.06	-0.03	-	-	0.05	0.07	-	-	0.04	0.08	-	-	0.10	0.21	-	-	0.06	-0.01	-	-
0.09	0.12	-	-	0.1	0.09	-	-	0.1	0.09	-	-	0.30	0.29	-	-	0.14	0.12	-	-
0.06	0.05	-	-	0.05	0.05	-	-	0.06	0.08	-	-	0.32	0.31	-	-	0.06	0.08	-	-
0.05	0.07	-	-	0.06	0.11	-	-	0.08	0.12	-	-	0.38	0.32	-	-	0.09	0.09	-	-
0.03	0.04	-	-	0.05	0.1	-	-	0.09	0.14	-	-	0.43	0.36	-	-	0.09	0.10	-	-
0.05	0	-	-	0.07	0.02	-	-	0.12	0.08	-	-	0.25	0.38	-	-	0.08	0.02	-	-
0.06	0.12	-	-	0.12	0.14	-	-	0.13	0.23	-	-	0.45	0.43	-	-	0.12	0.17	-	-
1.06	0.91	-	-	1.49	1.37	-	-	1.50	1.47	-	-	1.73	1.61	-	-	1.32	1.25	-	-
0.06	0.02	-	-	0.08	0.03	-	-	0.09	0.03	-	-	0.16	0.19	-	-	0.06	0.02	-	-
0.08	0.08	-	-	0.08	0.09	-	-	0.13	0.15	-	-	0.47	0.37	-	-	0.09	0.11	-	-

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