Determination of fat, protein and moisture in ricotta cheese by near infrared spectroscopy and multivariate calibration

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Abstract Near-infrared reflectance spectroscopy (NIRS) with partial least squares regression (PLSR) was used to determine levels of fat, protein and moisture in ricotta cheese without complex sample preparation. Spectra of 19 conventional and low-fat ricotta samples from different manufacturers were collected in duplicate, with 33 of the 38 spectra used as a calibration set and the remaining 5 spectra used as an external validation set. The best results were obtained by processing the spectral region between 1,100 and 2,500 nm. Multivariate models with six latent variables (LVs) showed good prediction capability for fat and protein determinations, with average relative errors (Er) of 6.37 % and 5.95 %, respectively. For moisture, a more robust model was obtained with 4 LVs, showing better prevision capacity and Er of 1.91 %.

Keywords Ricotta cheese · Fat · Protein · Moisture · Near infrared · PLSR

Introduction

Ricotta is a fresh cheese originating in Italy that is obtained by precipitating the proteins out of whey. Its production has increased, which is mainly because of consumers' search for healthy and low-calorie foods. The need to establish a standard of identity for ricotta cheese is essential for a better

control of product quality and consumer safety because there is not yet any Brazilian legislation specific to this cheese (Pintado et al. 2001; Esper et al. 2007).

According to current Brazilian legislation for cheeses, ricotta is classified using the identity and quality standards of low fat cheeses (Ministério de Agricultura e Reforma Agrária do Brasil 1996). However, Souza et al. (2000) evaluated 30 samples of ricotta, and found that 16.67 % of the samples could be classified as low-fat cheese, 23.33 % as semi-fat cheese and 60 % as full-fat cheese. This large variability justifies the need to establish identity and quality standards specific to ricotta, as well as to develop methodologies for rapid yet low-cost quality control (Esper et al. 2007; Cen and He 2007; Sultaneh and Rohm 2007).

Spectroscopy absorption in the infrared region has been valued for its high sensitivity, low cost, versatility and operational simplicity. Infrared spectroscopy provides a complete profile of the sample's composition, but it produces highly complex spectral signs and frequently has problems of non-linearity between concentration and absorption intensity; therefore, it does not easily allow quantitative applications (Reeves et al. 2001).

In the particular case of near-infrared spectroscopy (NIS), the majority of absorption bands are due to superpositions of different overtones and/or combinations of bands related to vibrational and rotational transitions. Large molecules and complex mixtures can therefore produce multiple bands and overlapping effects in the NIS spectrum, generating broad bands with few peaks (Karoui et al. 2003; Nicolaï et al. 2007).

The main disadvantages of the near-infrared spectroscopy have been circumvented by using multivariate calibration methods. Usually, this association dispenses sample pretreatments, which favors the development of easily-executed routines and the analysis of a large number of samples in few minutes (Hermida et al. 2001; Silva et al. 2009).

Partial least squares regression (PLSR) is a multivariate calibration method based on principal component analysis

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Table 1 Average chemical composition (% w/w), sample standard deviation (s) and sample coefficient of variation (CV) of ricotta samples analysed

Chemical composition	(% w/w)	s ^a	CV (%) ^b
Fat	10.63	± 5.14	48.35
Protein	12.37	± 2.92	23.61
Moisture	70.23	± 4.95	7.05
Total dry extract	29.77	± 4.95	16.63

^a Sample standard deviation: $s = (\sum(x_i - \bar{x})^2 / n - 1)^{1/2}$, summing the deviation in relation to the sample average, raised to the second power, divided by the number of samples minus one

^b Sample of coefficient of variation: (sample standard deviation/sample average) × 100

(PCA). This method does not restrict the wavelength number for calibration, making it possible to extract more information from the spectrum. After reduction of the dimensional space, the spectrum data (matrix X) are related to the concentration data (matrix Y), generating a regression coefficient vector (β) that best represents the matrix Y from the matrix X (Ferreira et al. 1999; Nagata et al. 2006). This methodology is especially indicated when matrix X contains highly correlated variables (as is the case with spectroscopy data), and it can be used even when the samples contain interfering signals (Caneca et al. 2006).

The use of near-infrared reflectance spectroscopy (NIRS) is recognized since the early sixties, when Hart et al. (1962) presented a method to determine moisture. Since then, many papers have described the use of NIRS in food analysis, including the quality control of wheat flour of milling industry (Miralbés 2004), the chemical composition of butter (Hermida et al. 2001), cocoa powder (Veselá et al. 2007), and grass silage (Villamarín et al. 2002), and the nutritional evaluation of cereals (Soares et al. 1998) and feed for rabbits (Xiccato et al. 1999, 2003). Work related to quality control of cheeses

Fig. 1 Principal component analysis scores using an MSC and MC from NIR spectra data to select a representative external validation set **Fig. 2** Diffuse reflectance near-infrared spectra of ricotta samples (calibration and validation sets)

include the determination of whey fat (Castillo et al. 2005), the determination of total solids and protein content in cheese curd (Sultaneh and Rohm 2007), the prediction of major components in commercial cheese (González-Martín et al. 2008), and the prediction of fatty acid composition of fresh and freeze-dried cheeses (Lucas et al. 2008).

This work emphasizes the need for specific regulatory identity standards for ricotta cheese in the Brazilian legislation to avoid the large observed discrepancy in fat and protein contents. To contribute to this issue, this paper proposes the use of diffuse reflectance spectroscopy associated with PLSR as a novel possibility for the quality control of ricotta cheese, particularly targeting the fast and inexpensive determination of fat, protein and moisture. (Kasimoglu et al. 2004; Pillonel et al. 2002).

Fig. 3 Root Mean Square Error of Cross Validation (RMSECV) with different number of latent variables for PLSR models for fat, protein and moisture determination by NIR

Fig. 4 Studentized residuals vs. leverage for identifying anomalous samples in moisture determination by NIR-PLSR

Materials and methods

Samples

Nineteen ricotta samples (including fresh, pressed, conventional, and low-fat varieties) manufactured in the southern and southeastern regions of Brazil were used to obtain the multivariate regression model. All samples were bought at local retail markets and refrigerated at 4 °C until analysis.

Reference methods

Ricotta samples were homogenized in a home pulverizing machine. The determination of fat (% w/w) was carried out using the Gerber van Gulik method (ISO 1975). The total nitrogen was determined using the Kjeldahl method and converted into protein percentage using the conversion factor 6.38 (the nitrogen-to-protein conversion factor for milk and dairy products) (IDF 1962). The moisture content and total dry matter of ricotta were determined by drying samples on a BRASIMET, Model ESE 35 stove at 105 °C for 12 h to achieve a constant weight (AOAC 1995). All analytical determinations were made in triplicate.

Near-infrared spectroscopy

Ricotta samples were cut to provide a flat surface that represented its interior. The flat surface of each sample was analyzed in a Near-Infrared Spectrophotometer (FEMTO, Model NIR 900 PLS) in duplicate. Each one of the duplicate spectra was obtained in a different zone of the flat surface. The spectra were obtained with 1 nm resolution in the spectral region from 1,100 to 2,500 nm. The instrument operated in a diffuse reflectance mode, with its signs expressed as log (1/R).

Data analysis

The spectral and concentration data were organized in matrices with Origin Software Pro 8.0, and the multivariate calibration model was developed with PLS-Toolbox 3.0 in Matlab 7.0.1.

The spectra were separated into two subsets: a calibration set (containing 33 spectra) and an external validation set (containing 5 spectra). This selection was made according to the PCA to obtain homogeneous and representative sets for calibration and validation.

The PLSR multivariate model was optimized using cross-validation (the leave-one-out approach). The performance of the resulting models was evaluated with respect to latent variables (LVs), cumulative variance, standard error or root mean square error of cross-validation (RMSECV), and cross validation correlation coefficient (Rval) for the calibration models. The ability of PLSR calibration models to estimate or predict the protein, fat and moisture levels from NIR spectra was confirmed by external validation.

Results and discussion

Table 1 contains the average results for the dry weight of the fat (% w/w), protein (% w/w), moisture (% w/w) and total dry matter (% w/w) in the 19 ricotta samples analyzed. There was large variability between samples: fat levels ranged from 1.84 % to 19.92 %, protein levels from 8.69 % to 17.97 %, moisture levels from 60.09 % to 81.35 % and total dry matter levels from 18.65 % to 39.91 %.

This large variability is in close agreement with the results found by Esper et al. (2007) in their study of the quality of ricotta samples commercialized in the city of Campinas, Brazil. The variations in the contents were as follows: 5.50 to 26.67 %

Fig. 5 Predictive vs. real values of fat (a), protein (b) and moisture (c) for samples of the calibration set

Table 2 Characteristics of optimized multivariate models to determination of fat, protein and moisture

	Transformation/preprocessing	VL ^a	Cumulative variance (%)		RMSEC/RMSECV (% m/m)	Rcal/Rval ^b
			X	Y		
Fat	MSC	6	100	98.7	1.3/1.9	0.968/0.936
Protein	MSC/MC	6	99.3	93.8	0.7/1.4	0.968/0.885
Moisture	Normalize	4	100	99.8	2.7/3.3	0.851/0.757

^a Number of latent variables^b Correlation coefficient–calibration set (Rcal) and cross-validation set (Rval)

w/w fat, 8.84 to 16.35 % w/w protein, 58.49 to 77.45 % w/w moisture and 22.55 to 41.51 % w/w total dry matter.

The fat and protein levels had the largest coefficients of variation, which can be attributed to the lack of standardization of milk fat levels both in the milk used for ricotta production and the milk added to the whey to improve the yield, taste and texture of the ricotta.

When evaluating the fat in dry matter (FDM = % fat × 100/% total dry matter (AOAC 1995)) according to the cheese classification system established by Regulation No. 146 (Ministério de Agricultura e Reforma Agrária do Brasil 1996), 5.26 % of the analyzed samples can be classified as skim cheese, 10.53 % as non-fat cheese, 57.89 % as semi-fat cheese and 26.32 % as full-fat cheese. All samples can be classified as very high moisture cheese according to Regulation No. 146, because they each have a moisture content over 55.0 %. Similar results were obtained by Souza et al. (2000) in their analysis of ricotta samples commercialized in the city of Belo Horizonte, Brazil. They showed that 16.67 % of the samples could be classified as non-fat cheese, 23.33 % as semi-fat cheese and 60 % as full-fat cheese. In addition, 3.33 % of the samples could be classified as medium moisture cheese, 3.33 % as high moisture cheese and 93.34 % as very high moisture cheese.

This wide variation in the composition of the commercialized ricotta samples is worrying because this product is frequently used by people with dietary restrictions, such as hypocaloric diets and diets for heart disease, high cholesterol and high triglycerides (Esper et al. 2007).

To obtain representative calibration and external validation sets despite this compositional variation, a PCA (Carvalho et al. 2000) was performed on the near-infrared spectral data of the ricotta samples. Multiplicative scatter correction (MSC) and data mean centering (MC) were used to obtain the score data of the first principal component (PC1) versus the second component (PC1 and PC2 represent 95.1 % of data variance–Fig. 1).

The highlighted samples in Fig. 1 were selected to compose the external validation set. The number of representative samples chosen from each quadrant was meant to be proportional to the total number of samples in each quadrant. The inclusion of isolated samples (high positive or negative scores), which could skew the data set, was avoided.

In this case, 33 spectra of ricotta samples were used for the calibration step, whereas five spectra were used for external validation. Figure 2 shows the spectra of ricotta samples.

For the construction of fat, protein and moisture models, the complete spectral range was used (from 1,100 to 2,500 nm).

Fig. 6 Vector of regression coefficients for PLSR models of fat, protein and moisture

Table 3 Prediction results of fat (%), protein (%) and moisture (%) concentration for the external validation sets

Samples	Real value (% w/w)			Predicted value (% w/w)			Relative error (%)		
	Fat	Protein	Moisture	Fat	Protein	Moisture	Fat	Protein	Moisture
1	13.05	12.77	67.69	12.76	13.46	66.19	−2.20	5.45	−2.22
2	13.05	12.77	67.69	14.15	13.12	67.97	8.46	2.73	0.41
3	9.89	16.02	68.88	8.83	15.14	67.57	−10.67	−5.47	−1.89
4	13.49	9.94	71.54	14.67	9.71	68.10	8.77	−2.36	−4.81
5	8.44	11.65	73.21	8.29	10.05	73.65	−1.77	−13.72	0.60
Mean relative error (%)							6.37	5.95	1.99

Different transformations and/or data preprocessing procedures were used to improve the efficiency of the regression models.

Fat and protein models used MSC for data transformation because this kind of treatment corrects the light spreading effects produced by the different reflections of analyzed surfaces. When constructing the models, it was observed the evolution of RMSECV values as a function of the number of LVs (Fig. 3). The RMSECV value increased beyond six LVs for the determination of protein or fat levels and beyond four LVs for moisture levels. Therefore, to avoid the aggregation of noise on the model, larger LV numbers were not used in the calibration set.

There is no evidence of anomalous samples in the fat and protein models. In the moisture model, spectrum 30 (Fig. 4–highlighted) has a high leverage value. The duplicate of this sample (spectrum 31), on the other hand, presents with a low leverage value. This discrepancy may indicate that spectrum 30 was obtained from a less flat surface compared with the duplicate spectrum, and that, for this reason, withdrawing spectrum 30 from the calibration set did not significantly change the model characteristics represented by LVs, RMSEC, RMSECV and correlation coefficients.

The models constructed showed strong correlations (Fig. 5) between values obtained by reference method and those obtained by multivariate model.

The characteristics of the more successful models are summarized in Table 2. The higher regression coefficients (Fig. 6) indicate the spectral regions of highest importance for the established correlation. The fat model was influenced mainly by the second overtone of C-H stretch at 1,190 nm (position 1), the combination vibration of C-H at 1,400 nm (position 2) and the first overtone of the C-H stretch at 1,710 nm (position 3) (Burns and Ciurczak 2008). In the protein model, position 4 indicates the first overtone C-N-C symmetric stretch (2,465 nm), while 5 and 6 are related to the absorbance of the N-H peptide (2,160 nm) and of amino acids (2,264 nm), respectively (Purnomoadi et al. 1999). In the case of moisture, the higher negative values for regression coefficients (*) are not directly related to the O-H bond. However, important spectral regions are indicated by positions 7 and 8, which are

combination bands involving O-H stretch and O-H bend, respectively. (Baddini et al. 2010)

The predicted values of fat, protein and moisture content in the external validation set samples are shown in Table 3. Samples one and two are actually the same ricotta sample with spectra obtained from two different parts of the analyzed surface. In other words, they obtained results provide evidence of the accuracy of multivariate spectrophotometric methodologies. It is worth mentioning that the greater similarity of the moisture level results might be due to a greater homogeneity of the moisture levels, which is evidenced by the lower VC value found during the characterization of ricotta samples (Table 1).

The fat, protein and moisture levels determined by multivariate methodology can be statistically compared using a paired *t*-test. No significant difference was found between the reference and multivariate methodologies using a significance level of 5 % ($t_{(v=4)} = 2.776$).

The multivariate models developed show similar prediction capacities to similar methods, using a lower number of LVs. For instance, Karoui et al. (2006) reported determination coefficients (R^2) of 0.92 for fat and 0.82 for total nitrogen quantification in samples of European Emmental cheese with NIRS. Its PLSR models were obtained with 10 and 7 LVs, respectively. Blazquez et al. (2004) reported even higher R^2 (0.99 for moisture using 4 LVs and 0.98 for fat using 5 LVs) in their analysis of processed cheese by NIRS.

Conclusions

The determination of fat, protein and moisture content in ricotta samples showed the lack of uniformity of the different ricotta brands, with greater coefficients of variation found for fat (48.35 %) and protein (23.61 %). As per Brazilian cheese classification system, 5.26 % of the analyzed cheese samples are considered skim, 10.53 % are non-fat, 57.98 % are semi-fat and 26.32 % are full-fat cheese. Regarding the moisture levels, all samples can be classified as high moisture cheese because they surpass the 55.0 % moisture limit proclaimed by the

Brazilian law. This great variability of data can be attributed to a lack of specific identity standards for ricotta cheese in the Brazilian legislation.

The multivariate models produced mean errors of prediction less than 6.4 %, with lower LV numbers than were found in the literature, showing that these are robust models with good predictive capacities. These results are evidence of the benefits of NIRS-PLSR, which allows rapid quality control with minimum manipulation of the sample.

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References

- AOAC (1995) Official methods of analysis of AOAC international. In: Official method 926.08. Moisture in cheese, 16 th edn. AOAC International, Maryland, vol II, pp 58
- Baddini ALQ, da Cunha LER, de Oliveira AMC, Cassella RJ (2010) Determination of total protein in hyperimmune serum samples by near-infrared spectrometry and multivariate calibration. *Anal Biochem* 397:175–180
- Blazquez C, Downey G, O'Donnell C, O'Callaghan D, Howard V (2004) Prediction of moisture, fat and inorganic salts in processed cheese by near infrared reflectance spectroscopy and multivariate data analysis. *J Near Infrared Spectrosc* 12:149–157
- Ministério de Agricultura e Reforma Agrária do Brasil (1996) Regulamentos técnicos de identidade e qualidade dos produtos lácteos. Diário Oficial da União de 11 março de 1996. In: Portaria nº 146, de 07 de março de 1996, pp 3977
- Burns AD, Ciurczak EW (2008) Handbook of near infrared analysis, 3rd edn. CRC Press Taylor & Francis Group, USA, pp 356–357
- Caneca AR, Pimentel MF, Galvão RKH, da Matta CE, De Carvalho FR, Raimundo IM Jr, Pasquini C, Rohwedder JJR (2006) Assessment of infrared spectroscopy and multivariate techniques for monitoring the service condition of diesel-engine lubricating oils. *Talanta* 70:344–352
- Carvalho RM, Mello C, Kubota LT (2000) Simultaneous determination of phenol isomers in binary mixtures by differential pulse voltammetry using carbon fibre electrode and neural network with pruning as a multivariate calibration tool. *Anal Chim Acta* 420:109–121
- Castillo M, Payne FA, Lopez MB, Ferradini E, Laencina J (2005) Preliminary evaluation of an optical method for modeling the dilution of fat globules in whey during syneresis of cheese curd. *Appl Eng Agric* 21:265–268
- Cen H, He Y (2007) Theory and application of near infrared reflectance spectroscopy in determination of food quality. *Trends Food Sci Technol* 18:72–83
- Esper LMR, Bonets PA, Kuaye AY (2007) Avaliação das características físico-químicas de ricotas comercializadas no município de Campinas-SP e da conformidade das informações nutricionais declaradas nos rótulos. *Rev Instituto Adolfo Lutz* 66:299–304
- Ferreira MMC, Antunes AM, Melgo MS, Volpe PLO (1999) Quimiometria I: calibração multivariada, um tutorial. *Quím Nova* 22:724–731
- González-Martín I, González-Pérez C, Hernández-Hierro JM, González-Cabrera JM (2008) Use of NIRS technology with a remote reflectance fibre optic probe for predicting major components in cheese. *Talanta* 75:351–355
- Hart JR, Norris KH, Golumbic C (1962) Determination of the moisture content of seeds by near-infrared spectrophotometry of their methanol extracts. *Cereal Chem* 39:94–99
- Hermida M, Gonzalez JM, Sanchez M, Rodriguez O, Jose L (2001) Moisture, solids-non-fat and fat analysis in butter by near infrared. *Int Dairy J* 11:93–98
- IDF (1962) Determination of the total nitrogen content of milk by the Kjeldahl method. In: International Standard 20, International Dairy Federation, Belgium
- ISO (1975) Cheese. Determination of fat content. Van Gulik method. In: Standard 3433, International standards organization, Switzerland
- Karoui R, Mazerolles G, Dufour E (2003) Spectroscopic techniques coupled with chemometric tools for structure and texture determinations in dairy products. *Int Dairy J* 13:607–620
- Karoui R, Mouazen AM, Dufour É, Pillonel L, Schaller E, Baerdemaeker JD, Bosset JO (2006) Chemical characterisation of European Emmental cheese by near infrared spectroscopy using chemometric tools. *Int Dairy J* 16:1211–1217
- Kasimoglu A, Göncüoglu M, Akgün S (2004) Probiotic white cheese with *Lactobacillus acidophilus*. *Int Dairy J* 14:1067–1073
- Lucas A, Andueza D, Ferlay A, Martin B (2008) Prediction of fatty acid composition of fresh and freeze-dried cheeses by visible–near-infrared reflectance spectroscopy. *Int Dairy J* 18:595–604
- Miralbés C (2004) Quality control in the milling industry using near infrared transmittance spectroscopy. *Food Chem* 88:621–628
- Nagata N, Peralta-Zamora P, Poppi RJP, Perez CA, Bueno MIMS (2006) Multivariate calibration for the SR-TXRF determination of trace concentrations of lead and arsenic in the presence of bromine. *X-Ray Spectrom* 35:79–84
- Nicolai BTM, Beullens K, Bobelyn E, Peirs A, Saeys W, Theron KI, Lammertyn J (2007) Nondestructive measurement of fruit and vegetable quality by means of NIR spectroscopy: a review. *Postharvest Biol Tec* 46:99–118
- Pillonel L, Badertscher R, Bütikofer U, Casey M, Dalla Torre M, Lavanchy P, Meyer J, Tabacchi R, Bosset J (2002) Analytical methods for the determination of the geographic origin of Emmentaler cheese. Main framework of the project; chemical, biochemical, microbiological, colour and sensory analyses. *Eur Food Res Technol* 215:260–267
- Pintado ME, Macedo AC, Malcata FX (2001) Review: technology, chemistry and microbiology of whey cheeses. *Food Sci Technol Int* 7:105–116
- Purnomoadi A, Batajoo KK, Ueda K, Terada F (1999) Influence of feed source on determination of fat and protein in milk by near-infrared spectroscopy. *Int Dairy J* 9:447–452
- Reeves JB, McCarty GW, Reeves VB (2001) Mid-infrared diffuse reflectance spectroscopy for the quantitative analysis of agricultural soils. *J Agric Food Chem* 49:766–772
- Silva FEB, Ferrão MF, Parisotto G, Muller EI, Flores EMM (2009) Simultaneous determination of sulphamethoxazole and trimethoprim in powder mixtures by attenuated total reflection-Fourier transform infrared and multivariate calibration. *J Pharm Biomed* 49:800–805
- Soares AMB, Murray I, Paterson RM, Abreu JMF (1998) Use of near infrared reflectance spectroscopy (NIRS) for the prediction of the chemical composition and nutritional attributes of green crop cereals. *Anim Feed Sci Technol* 75:15–25
- Souza MR, Morais CFA, Corrêa CES, Rodrigues R (2000) Características Físico-Químicas de ricotas comercializadas em Belo Horizonte, MG. *Rev Hig Aliment* 14:68–71
- Sultaneh A, Rohm H (2007) Using near infrared spectroscopy for the determination of total solids and protein content in cheese curd. *Int J Dairy Technol* 60:241–244
- Veselá A, Barros AS, Synytsya A, Delgadillo I, Copiková J, Coimbra MA (2007) Infrared spectroscopy and outer product analysis for

- quantification of fat, nitrogen, and moisture of cocoa powder. *Anal Chim Acta* 601:77–86
- Villamarín B, Fernández E, Mendez J, Coeem SCL (2002) Analysis of grass silage from Northwestern Spain by near-infrared reflectance spectroscopy. *J AOAC Int* 85:541–545
- Xiccato G, Trocino A, Carazzolo A, Meurens M, Maertens L, Carabaño R (1999) Nutritive evaluation and ingredient prediction of compound feeds for rabbits by near-infrared reflectance spectroscopy (NIRS). *Anim Feed Sci Technol* 77:201–212
- Xiccato G, Trocino A, De Boever JL, Maertens L, Carabaño R, Pascual JJ, Perez JM, Gidenne T, Falcao-Ecunha L (2003) Prediction of chemical composition, nutritive value and ingredient composition of European compound feeds for rabbits by near infrared reflectance spectroscopy (NIRS). *Anim Feed Sci Technol* 104:153–168