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Characterization and detection of adulterated whey protein supplements using stationary and time-resolved fluorescence spectroscopy

Cristina Guimarães Pereira[a,*], Jonathan Andrade[a,**], Thamiris Ranquine[a], Israel Novaes de Moura[a], Roney Alves da Rocha[b], Marco Antônio Moreira Furtado[c], Maria Jose Valenzuela Bell[a], Virgilio Anjos[a]

[a] Grupo de Engenharia e Espectroscopia de Materiais, Departamento de Física, Universidade Federal de Juiz de Fora, Rua José Lourenço Kelmer, s/n - São Pedro, 36036-900, Juiz de Fora, MG, Brazil
[b] Departamento de Ciência de Alimentos, Universidade Federal de Lavras, Caixa Postal 3037, 37200-000, Lavras, Minas Gerais, Brazil
[c] Departamento de Ciências Farmacêuticas, Faculdade de Farmácia, Universidade Federal de Juiz de Fora, Rua José Lourenço Kelmer, s/n - São Pedro, 36036-900, Juiz de Fora, MG, Brazil

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ABSTRACT

The objective of this work was to evaluate the efficacy of time-resolved and stationary fluorescence spectroscopic techniques in the detection of adulterations in Whey Protein Concentrate (WPC) powder, used in nutritional supplements. Adulterations were performed by the individual addition of caffeine, creatine and lactose in WPC in different levels (10%, 20% and 30% w/w). Samples were submitted to 275 nm excitation wavelength. Time-resolved fluorescence revealed that the adulterations changed the lifetimes of the tri-exponential decay curves obtained at 335 nm. The 30% adulterations demonstrated higher values of the mean emission lifetime, with caffeine treatment statistically significant. The fluorescence spectra showed an emission peak at approximately 335 nm with a broadband between 325 and 430 nm in the caffeine treatment. Euclidean distance analysis indicated the creatine and lactose treatments (10, 20 and 30% w/w) as similar to control WPC, while caffeine 20% and 30% treatments not. Hierarchical Cluster Analysis could differentiate all caffeine treatments from the other samples. Principal Component Analysis confirmed the differentiation of all adulterants from the control WPC. In general, the application of time-resolved fluorescence and stationary fluorescence spectroscopy techniques allowed the characterization and observation of differences among the samples, when allied to statistical tools.

1. Introduction

Milk whey is defined as a by-product of cheese-making and it was historically considered as a waste product and environmental pollutant, with no commercial use (Walzem, Dillard, & German, 2002). Whey constitutes about 85–90% of the volume of the milk used in cheese production and it retains about 55% of the milk nutrients (Sinha, Radha, Prakash, & Kaul, 2007). Thus, innumerous technical and nutritional applications were found with examples of their usage in processed meat, bakery, dairy products, nutritional supplements and so on (Moro, Gatti, & Delorenzi, 2001). As a functional ingredient, it has strong application in the sports nutrition market, since it is rich in essential amino acids, especially those with branched side chains (Bos, Gaudichon, & Tomé, 2000; Walzem et al., 2002). The whey protein concentrate (WPC) is used as protein-based food supplement by sportsmen and athletes with claims of higher yield in protein anabolism and enhanced performance.

Throughout the years, the public awareness regarding food quality and safety has been increasing, and also the food authentication field, which verifies if a given food is following its label description. Thus, the process of continuous quality monitoring is an indisputable requirement of the modern food industry (Danezis, Tsagkaris, Camin, Brusic, & Georgiou, 2016; Reid, O'Donnell, & Downey, 2006). Since whey value has grown to a commodity level and due to the recent growing consumption of these supplements, it has been target for adulteration with cheaper components products (Garrido, Souza, Lourenço, & Fasciotti, 2016). Carbohydrates, amino acids derivatives and thermogenic substances are common compounds used as substitutes. Lactose, the main carbohydrate of milk, contributes approximately with 70% of the content of whey powder (Moriwaki & Matiolo, 2000) and it is commonly detected in many adulterated powders (Ordóñez, Rodriguez, & Sanz, 2005). Creatine is a nitrogenous organic acid that facilitates the

* Corresponding author.
** Corresponding author.
E-mail addresses: crisgp13@yahoo.com.br, cristinaengcivil@hotmail.com (C.G. Pereira), dr.andradej@gmail.com, larvax@gmail.com (J. Andrade).

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recycling of adenosine triphosphate in muscles, improving physical performance, being widely used by bodybuilders to increase strength and muscle mass (Bemben & Lamont, 2005). Among the thermogenic compounds, the main example is caffeine, which stimulates the nervous system and increases the mobilization of free fatty acids to the muscles improving endurance performance during prolonged exercise (Braga & Alves, 2000).

Several food products have been subjected to falsification including milk, milk products, wines, coffee, chocolate, and many others (Alamprese, Casale, Sinelli, Lanteri, & Casiraghi, 2013; Danezis et al., 2016; Lerma-García, Ramis-Ramos, Herrero-Martínez, & Simó-Alfonso, 2010; Siddiqui, Musharraf, Choudhary, & Rahman, 2017). Attempts using spectroscopic methodologies to investigate authenticity issues have been done using mainly the UV-Visible and the Fourier Transform Near Infrared (NIR), Mid Infrared (MIR) and Raman spectroscopies (Alves da Rocha, Paiva, Anjos, Furtado, & Bell, 2015; Di Anibal, Rodriguez, & Albertengo, 2014; Haughey, Galvin-King, Ho, Bell, & Elliott, 2015; Mendes et al., 2015). With advantages of being non-destructive, environmental friendly, and hold relatively low cost of analysis, the spectroscopic analysis can provide accurate qualitative and quantitative data (Andrade et al., 2018; Danezis et al., 2016; Lohumi, Lee, Lee, & Cho, 2015). Other spectroscopic techniques not much commonly used so far, but with an incredible potential, are the stationary and time-resolved fluorescence spectroscopy, with versatile applications (Lemos, Aliyu, & Hungerford, 2012) that allow the characterization of dairy products in relation to authenticity, security and detection of adulterations (Brandao, de Carvalho dos Anjos, & Bell, 2017a; Brandao, dos Anjos, & Bell, 2017b; Brandao, Gouvea Neto, dos Anjos, & Bell, 2017c).

When food samples are submitted to fluorescence measurements, the components within the food matrix are responsible for the emission obtained. In dairy products, several substances contribute to fluorescence such as riboflavin, vitamin A, aromatic amino acids, Maillard reaction and lipid oxidation products, coenzymes (e.g. NADH), among others. Each molecule within the powder mixture has its characteristic excitation and emission spectra, which can be used to separate and identify adulterations and to differentiate substitutions (Andersen & Mortensen, 2008).

To date, there is no research work undertaken attesting the authenticity of whey protein supplements using fluorescence spectroscopy. Therefore, this study assessed the potential application of the stationary and time-resolved fluorescence spectroscopy to characterize and detect adulteration of WPC, by addition of caffeine, creatine and lactose. Also, it was used the multivariate tool of Hierarchical Cluster Analysis (HCA) and Principal Component Analysis (PCA) to allow a better understanding and characterization of the adulterated samples compared to the pure WPC.

2. Material and methods

2.1. Acquisition and preparation of samples

WPC (Infinitypharma, Nova Iguaçu, RJ, Brazil) was acquired in the local market of the city of Juiz de Fora, MG - Brazil. Adulterations were made by adding caffeine (Fagron do Brasil Farmacêutica Ltda., São Paulo, SP, Brazil), lactose monohydrate (Henrifarma Produtos Químicos e Farmacêuticos, São Paulo, SP, Brazil) and creatine (Infinitypharma, Nova Iguaçu, RJ, Brazil) into WPC powder.

The experiment consisted in the production of 78 tablets of 130 mg of pure components (WPC from 2 different batches, caffeine, creatine and lactose) and also mixtures of the adulterants in WPC in proportions of 10%, 20% and 30% w/w. The WPC batches were used to carry out distinct experiments. In each experiment, 12 tablets were produced with pure components and 27 with mixtures of WPC and the correspondent adulterant. All analyses were carried out in triplicate. Once nutritional supplements such as WPC contains 65–80% of protein on a dry basis (Tunick, 2008), the maximum adulterant concentration of 30% was chosen since the reduction of 30% on the total amount of WPC in the sample surely brings its protein content below 65% (on a dry basis), characterizing a fraudulent product. Therefore, the samples were weighted, homogenized with mortar and pestle, and submitted to a hard-press treatment using an automatic hydraulic press (Atlas™ Power Hydraulic Press T25, SPECAC INC, Fort Washington, PA, USA) set to 3 tons during 1 s, to create a tablet with 1 cm of diameter and thickness of 1 mm.

2.2. Time-resolved fluorescence spectroscopy

Preliminary analysis with five different fixed excitation wavelengths (275, 290, 305, 320 and 335 nm) was carried out to select the one that induced the highest emission intensity of WPC components. The light source at 275 nm has shown this feature, generating a fluorescent emission at 335 nm. The fluorescence intensity decay was measured on basis of time-correlated single photon counting (TCSPC), using an ultrafast nanosecond flashlamp (nF920 Edinburgh Instruments, Livingston, UK) filled with hydrogen gas as an excitation source, with pressure between 0.4 and 0.7 bar, frequency of 40 kHz and voltage of 6.8 kV. The photon emission acquisitions were performed with a spectrometer (FL920 model, Edinburgh Instruments, Livingston, UK), with a blue-sensitive photomultiplier.

The data were fitted by performing a deconvolution of all decays with the instrument response function. The reliability of the fit was acquired through the reduced chi-square using the software F900 (Edinburgh Instruments, Livingston, UK). Eq. (1) shows the fluorescence intensity decay over time (Brandao et al., 2017b), where β_i are the pre-exponential factors and τ_i are lifetimes (ns).

$$I(t) = \sum_i \beta_i \exp(-t/\tau_i) \quad (1)$$

Throughout the experimental decay fitting results, the mean intensity lifetime was calculated by Eq. (2) (Brandao et al., 2017b), where τ is the mean intensity lifetime (ns).

$$\tau = \sum_i \beta_i \tau_i^2 / \sum_i \beta_i \tau_i \quad (2)$$

2.3. Stationary fluorescence spectroscopy

The emission spectra were obtained using the spectrometer (FL920 model, Edinburgh Instruments, Livingston, UK), with a blue-sensitive photomultiplier to detect the emitted photons. The data were processed with the F900 software (Edinburgh Instruments, Livingston, UK). The excitation source was the Edinburgh nF900 ultrafast nanosecond flashlamp filled with hydrogen gas. The exposure time to the source was 0.1 s, and for each emission, the result was the sum of three scans. The emission maps were obtained at 275 nm excitation wavelength acquiring emission intensities from 300 nm to 527 nm range, with 1 nm step.

2.4. Statistical treatments

2.4.1. Time-resolved fluorescence spectroscopy

The data obtained were submitted to analysis of variance (One-way ANOVA) and Tukey HSD test. All statistical tests were done with 95% of significance using the free software [R] (www.r-project.org).

2.4.2. Stationary fluorescence spectroscopy

The analysis of similarity measures the distance among the discretized emission signals of two samples. To investigate a given similarity, it was used the Euclidean Distance (ED). An ellipse of standard deviation can provide the knowledge of a spatial dispersion of data (Altman & Bland, 2005; Tartaruga, 2008). The standard deviation