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A fast semi-quantitative screening for cocoa content in chocolates using MALDI-MSI

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ABSTRACT

Chocolate is a popular food bearing a number of different classifications that are differentiated by proportions of cocoa solids, milk and cocoa butter. Literature brings evidence that chocolates with a high percentage of cocoa solids contribute to good health maintenance due to the presence of phenolic compounds. On the other hand, it is known that the productive process, including pre-processing, may influence the level of these substances in the finished product. Thus, accurate strategies to measure the levels of this class of molecules that can be highly adaptable throughout the manufacturing process are important to ensure high-quality products. Mass spectrometry is an analytical tool of high sensitivity and specificity that is leading the research in food analysis towards new directions. By using mass spectrometry imaging in direct food analysis, this contribution developed an effective methodology for comparatively establishing the levels of catechin/epicatechin as phenolics content markers for cocoa content in a series of commercial chocolates from a single manufacturer, rendering a versatile tool that can be applied in fast screening of cocoa content in finished products and during manufacturing.

1. Introduction

Chocolate is a food product that is commercially classified by the cocoa content declared by manufacturers. This results in four main different types of product, namely bitter, semisweet, milk, and white, which present varying amounts of cocoa solids, milk, sugar and cocoa butter (de Oliveira, Castro, de Oliveira, & de Oliveira, 2015), in addition to different resulting proportions of carbohydrates, fat and protein contents. Additionally, other classes of compounds such as minerals and phenolics are present in variable concentrations, depending on parameters such as cocoa percentage.

Cocoa and derived food products present, as a general rule, considerable flavonoid content, with highlights to flavan-3-ols, especially catechin/epicatechin (Hu et al., 2016). The health benefits of these bioactive molecules in humans has been widely studied, especially a number of protective implications over the cardiovascular system (Pereira et al., 2014) and the prevention of cognitive function decay with aging (Sokolov, Pavlova, Klosterhalfen, & Enck, 2013).

With abundant information on food products available over the internet and other sources, consumers are more prone to choose their products by relying on labels that contain ingredients or contents that meet their own criteria (Bakke, Shehan, & Hayes, 2016). Therefore, assessing the levels of different ingredients in chocolate for quality purposes is critical from a commercial point of view, as providing evidence to consumers that they are purchasing the right product based on their preference has transcended the economic aspect only. Thus, establishing accurate and timely strategies for both quality control and supplier certification have become even more relevant for companies.

There is a variety of analytical methods in the literature that assess different aspects of chocolate; Fourier-transform infrared spectroscopy (FTIR), for instance, has shown great potential in the evaluation of adulterations in fat content (Che Man, Syahariza, Mirghani, Jinap, & Bakar, 2005), as did gas chromatography coupled with mass spectrometry (GC–MS) for volatile compounds (Afoakwa, Paterson, Fowler, & Ryan, 2009). Quality assessment of cocoa content in chocolates, however, is still impaired by the lack of diversity and versatile methods that encompass both qualitative and quantitative approaches in literature. Additionally, there is no consensus in suitable markers/molecules to describe this component, due to variations in the quali-quantitative chemical composition of cocoa beans and mass according to geographic origin, environmental factors, processing, fermentation, etc. (Carrillo, Londoño-Londoño, & Gil, 2014). While chromatographic approaches are being widely used for phenolics in chocolates (Alañón, Castle, Siswanto, Cifuentes-Gómez, & Spencer, 2016; Pedan, Fischer, Bernath, Hühn, & Rohn, 2017) with consistent quantitative results, alternatives aimed at saving both analysis time and solvent consumption are being sought. Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry is ultimately emerging as a potentially suitable

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method for assessing cocoa content and quality through the evaluation of phenolics (Bonatto & Silva, 2015). Developing analytical strategies that are able to provide fast and reliable results regarding quali-quantitative aspects are therefore a growing trend in determining the quality of commercial chocolates through cocoa content.

In this sense, mass spectrometry imaging (MSI) has been emerging as an important analytical tool that is robust enough to provide not only qualitative, but also quantitative aspects in solid-state samples, with minimal preparation requirements (de Menezes, de Oliveira, & Catharino, 2016; de Oliveira, Ferreira, & Catharino, 2014). MSI demonstrates great versatility, due to the combination of a highly-specific mass analyzer that provides accuracy through MS/MS reactions, and the analytical approach flexibility provided by offering either direct relative quantification between a set samples with similar characteristics, and absolute quantification that allows a fixed validated process to be implemented. This translates into enhanced selectivity in quality markers, for example phenolic compounds that are specific for chocolate but may present variable amounts across manufacturers and producers, thereby tackling an important issue regarding chemical composition of natural products in foods. The aim of this contribution, therefore, was to explore this feature in actual chocolate samples, assessing cocoa content by providing the relative quantification profile of catechin/epicatechin in a series of chocolate samples from the same manufacturer, but with different declared cocoa content. Our results indicated that this approach is not only capable of yielding a largely versatile method that can be tailored according to the user's need, but it was also understood as an expeditious screening method for in-process evaluation of the quality parameter of cocoa content during virtually any step of chocolate manufacturing process chain.

2. Materials and methods

2.1. Chocolate samples

Commercially available samples of chocolate at five different declared percentages of cocoa content in the label were purchased from a certified Brazilian manufacturer. The percentages used for this study were 28%, 41%, 55%, 70% and 85%. Cocoa liquor, obtained from the same manufacturer, was used as the 100% standard for comparison purposes. Samples of milk, cocoa butter, and MALDI matrix were also analyzed as blanks to asses any potential interference in the results. Ten samples were analyzed for each studied condition (n = 10).

2.2. Sample preparation

Samples were imprinted onto a silica plate (Merck, Darmstadt, Germany), covered with a 10-mg/mL solution of MALDI matrix α-cyanohydroxycinnamic acid (Sigma-Aldrich, St. Louis, MO) in MeOH:AcN (1:1), and sent for direct analysis, in a similar approach as developed by de Oliveira et al. (2014). A general scheme of the workflow is depicted in Fig. 1. All ten replicates for each cocoa percentage were prepared individually, according to the abovementioned workflow, and analyzed across different days.

2.3. MALDI-MSI analyses

The phenolic species catechin/epicatechin were elected as the selective quality markers for cocoa content. An LTQ-XL MALDI instrument (Thermo Scientific, San Jose, CA) was used to monitor the $[M + H]^+$ ion at *m/z* 291 after characterization through MS/MS reactions, compared to the fragments from a catechin standard (Sigma-Aldrich, St. Louis, MO) and calculated collision-induced dissociation products using the Mass Frontier software (Thermo Scientific, San Jose, CA). Chemical images with 600 square pixels were generated through selection of desired areas in the silica plate in the software prior to analyses. Images were then processed in grayscale using the ImageQuest software (Thermo Scientific, San Jose, CA), and ultimately cropped and submitted for relative quantification using the open-source software ImageJ (US-NIH, Bethesda, MD), in an adaptation of de Oliveira, de Bona Sartor, Ferreira, and Catharino (2013).

2.4. Statistical analyses

A Kolmogorov-Smirnov normality test was performed to assess whether samples presented normal distribution in each concentration percentile, using GraphPad Prism software (v. 5.01, GraphPad Software, San Diego, CA, USA). The same software was used to assess and plot the relative quantification data to obtain the Pearson r-value, R-squared, and the associated two-tailed p–value.

3. Results and discussion

MALDI-MSI was utilized in the MS/MS mode, i.e. only the selective ion at *m/z* 291 $[M + H]^+$ was monitored during the analyses. An ion was considered accurate for monitoring when the transitions observed after MS/MS reactions matched those from the catechin standard, i.e. *m/z* 275, 247 and 197; these main product ions were therefore used for characterization purposes, and matched the in silico calculations for MS/MS fragmentation patterns using Mass Frontier. Additionally, to ensure that the selected specie was characteristic of cocoa only, both the parent and product ions were tested for blank samples (milk, cocoa butter and MALDI matrix), wherein their absence was confirmed by presenting spectral intensities below the signal-to-noise ratio (data not shown). This strategy is categorical in determining the specificity of the target ions; as the instrument is set to acquire only the desired species, there is little or no chance of interference from other existing species in the sample, which is consistent with a method that is intended and designed to provide accurate results in cocoa content. Since chocolate is a complex sample that is comprised of several molecular classes, the monitoring of a single ion may pose a challenge due to ionization issues, ion suppression, etc., which are eliminated by this feature (Knochenmuss, Dubois, Dale, & Zenobi, 1996).

To explore the semi-quantitative aspect of MSI, ImageJ software assigns non-dimensional values to images, similarly to the area under the curve (AUC) values obtained with chromatographic approaches. Comparatively, the higher the amount in a sample that undergoes chromatography, the higher will be the AUC value; in contrast, ImageJ provides higher values for darker pixels, indicating that this intensity variation is directly proportional to the amount of the selected ion in

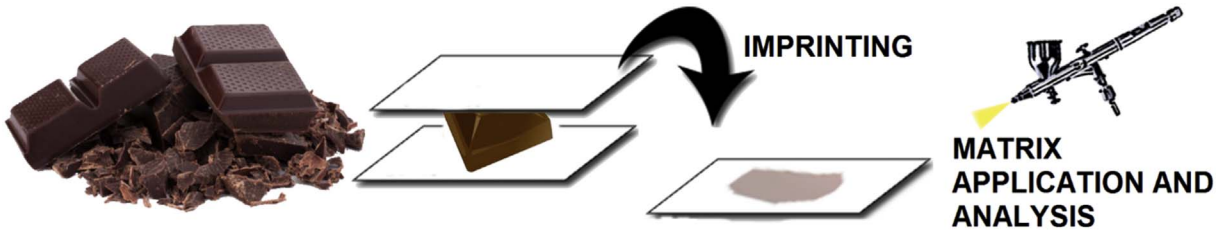

Fig. 1. A simplified workflow for the preparation of chocolate samples followed by MALDI-MSI analyses.