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Analytical Methods

Multiresponse optimization of a UPLC method for the simultaneous determination of tryptophan and 15 tryptophan-derived compounds using a Box-Behnken design with a desirability function

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Indole-3-carboxylic acid (PubChem CID: 69867)
5-Methoxytryptamine (PubChem CID: 6198)
Indole-3-acetonitrile (PubChem CID: 351795)
5-Methoxy-DL-tryptophan (PubChem CID: 119802)
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ABSTRACT

A Box–Behnken design was used in conjunction with multiresponse optimization based on the desirability function to carry out the simultaneous separation of tryptophan and 15 derivatives by Ultra Performance Liquid Chromatography. The gradient composition of the mobile phase and the flow rate were optimized with respect to the resolution of severely overlapping chromatographic peaks and the total run time. Two different stationary phases were evaluated (hybrid silica and a solid-core-based C_{18} column). The methods were validated and a suitable sensitivity was found for all compounds in the concentration range 1–100 μg L^{-1} ($R^2 > 0.999$). High levels of repeatability and intermediate precision (CV less than 0.25% and 1.7% on average for the retention time and the signal area, respectively) were obtained. The new method was applied to the determination tryptophan and its derivatives in black pigmented glutinous and non-glutinous rice grain samples.

1. Introduction

Tryptophan is one of the essential amino acids in human nutrition and it is considered to be exceptional in its diversity of

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biological functions in both the animal and plant kingdoms (Ren, Zhao, Wang, Cui, & Yang, 2007). This indole-containing compound serves as a biochemical precursor for substances associated with typical physiological activity, including vitamins, neurotransmitters and hormones (Fukuwatari & Shibata, 2013).

The identification of indole-3-acetic acid (IAA), the tryptophan-derived phytohormone discovered in 1934 (Mano & Nemoto, 2012), has led to a longstanding interest in major research on plant growth regulators and their applications in biotechnology. In 1948 another tryptophan-derived compound that is structurally similar to IAA, namely serotonin (5-hydroxytryptamine; 5HT), was found to coexist in bovine serum with tryptophan (Rapport, Green, & Page, 1948). Half a century later this substance was also identified in plants (Grobe, 1982) and it was demonstrated to be a phytohormone in view of its auxin-like activity (Ramakrishna, Giridhar, & Ravishankar, 2011).

Several related compounds that share common biosynthetic pathways with IAA and 5HT, e.g., melatonin (*N*-acetyl-5-methoxytryptamine; NA5MT), tryptamine (TAM), indole-3-acetonitrile (IAN) and indole-3-butyric acid (IBA), contribute to a possible coordinated regulation in plants (Arnao & Hernández-Ruiz, 2006; Zhao, 2012). Kynurenine has recently been reported to constrain competitively a class of tryptophan aminotransferases (Mashiguchi et al., 2011) and, as a consequence, chemical inhibition of the IAA biosynthesis pathway has also been used to support research into plant growth regulation (He et al., 2011). Interactions between tryptophan-derived compounds are critical for plants to adjust to environmental changes.

Previous and current analyses of tryptophan-derived compounds have led to the wide acceptance of the notion that crucial biological functions cannot be fully elucidated by a mono-causal approach. Nonetheless, tryptophan-derived compounds seems to be essential to comprehend their functions rather than the absolute levels of any single substance (Wang & Irving, 2011). Therefore, a sensitive and selective method for the simultaneous determination of tryptophan and its derivatives is a prerequisite for analytical applications and physiological research.

The biggest obstacle to the simultaneous determination of tryptophan metabolites is the fact that the substances are structurally very similar and they therefore have very similar absorption spectra in the UV wavelength range (Data included in the Supplementary Material). The challenge of developing a suitable analytical method for the determination of tryptophan-derived compounds has already been addressed using different detection and separation systems (Cao, Murch, O'Brien, & Saxena, 2006; Giannarelli et al., 2010; Kato et al., 2007; Ma et al., 2008; Vignau, Jacquemont, Lefort, Imbenotte, & Lhermitte, 2004; Zagajewski et al., 2012). However, many of the reported methods were focused on either multi-parallel analysis or on improved sensitivity without addressing the selectivity of detection and, furthermore, the methods were relatively time-consuming. Moreover, the number of compounds studied in a single analysis was still very limited. Hence, the ultimate aim of the study reported here was to achieve a reliable, efficient, sensitive and rapid multi-targeted quantification of tryptophan-derived compounds in a single analysis run.

The analytical platform of choice for this study was ultra-performance liquid chromatography (UPLC) coupled to a photo-diode array (PDA) detector because this chromatographic technique can achieve dramatic increases in resolution, speed and sensitivity in liquid chromatography. In UPLC the negative characteristics associated with packed columns used in high-performance liquid chromatography (HPLC) have been overcome by delivering the mobile phase precisely at pressures up to 15,000 psi (1030 bar), thus enabling the use of shorter columns packed with smaller particles (sub-2 μm) to provide a higher level of chromatographic performance.

A hybrid-silica technology has been developed in an effort to ensure that UPLC columns have sufficient mechanical strength. A particle size of 1.7 μm was selected for the column design along with bridging of the methyl groups in the silica matrix to obtain enhanced mechanical stability for use over a wide pH and temperature range. Additionally, solid-core silica technology was designed in order to achieve maximum efficiency. As the name implies, this system has a non-porous solid-core of silica in the center of the particle and this is surrounded by an outer layer of porous silica, on which the chromatographic separation takes place.

Although solid-core-based columns have lower pore volumes and lower surface areas than the corresponding particle-based columns, the calculated phase ratios for the two types of material are similar as a result of their equivalent retention factors. Therefore, in order to evaluate the separation efficiency between these two types systems, columns with matching dimensions (100 mm length; 2.1 mm I.D.) were evaluated in this study. In addition to the enhancements in resolution and peak efficiency (peak width) on using columns with smaller particles, which leads to shorter analysis times, UPLC technology requires smaller amounts of solvents compared with conventional HPLC (Nováková, Matysová, & Solich, 2006). Furthermore, the UPLC system allows a low limit of detection since the signal-to-noise ratio is improved and the injection volume can be reduced considerably without a loss of sensitivity (Srivastava, Sharma, Baghel, Yaswant, & Sethi, 2010). Nonetheless, method development for UPLC is required in order to identify the optimum conditions for full separation.

The most important aspect of method development in liquid chromatography is the simultaneous optimization of the resolution and analysis time (Hadjmohammadi & Sharifi, 2012), both of which depend on several variables such as gradient time and flow rate (Coutinho et al., 2015). Additionally, the composition of the mobile phase and the type of column are also important variables when optimizing a UPLC separation (Pan, Li, Li, Chen, & Bai, 2014). In the study reported here, the effects of several variables were evaluated: namely the effects that composition of the mobile phase at the start and end of the gradient program and the flow rate had on the resolution and analysis time.

The optimization process was developed by carrying out experiments based on a Box–Behnken design (BBD) (Zhou et al., 2010, 2009, Pedro, Moreira, Granato, & Rosso 2016). For three variables, as defined in this study, the Box–Behnken design offers some advantages in that it requires fewer runs other experimental designs (Ferreira et al., 2007; Pedro, Granato, & Rosso 2016b). However, when the optimization procedure involves more than one response, it is not possible to optimize each one in a separate way, because a number of solutions equal to the number of variables under study would be generated (Candioti, De Zan, Cámara, & Goicoechea, 2014). In this case, the overall solution must be included in an optimal region, leading to a certain degree of compliance with the proposed criteria for each variable of the system. A desirability function can be employed to find a compromise solution for multiresponse optimization (MRO) (Granato, Grevink, Zielinski, Nunes, & van Ruth 2014; Islam, Alam, & Hannan, 2012).

The objective of the study reported here was to optimize the UPLC method for the simultaneous separation of tryptophan and 15 tryptophan derivatives using a BBD in conjunction with MRO and a desirability function. As part of the method validation process, the optimized method was applied to rice grain matrices using a previously published extraction and clean-up procedure for a specific tryptophan-derived compound (Setyaningsih, Duros, Palma, & Barroso, 2016).