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Study of ion suppression for phenolic compounds in medicinal plant extracts using liquid chromatography–electrospray tandem mass spectrometry

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ABSTRACT

A systematic study on the various sources of ion suppression in UHPLC-MS–MS analysis was carried out for 24 phenolic antioxidants in 6 different extracts of medicinal plants from Amazonia. The contributions of matrix effects, mobile-phase additives, analyte co-elution and electric charge competition during ionization to the global ion suppression were evaluated. Herein, the influence of mobile-phase additives on the ionization efficiency was found to be very pronounced, where ion suppression of approximately 90% and ion enhancement effects greater than 400% could be observed. The negative effect caused by the wrong choice of internal standard (IS) on quantitative studies was also evaluated and discussed from the perspective of ion suppression. This work also shows the importance of performing studies with this approach even for very similar matrices, such as varieties of medicinal plants from the same species, because different effects were observed for the same analyte.

1. Introduction

Medicinal plants and foodstuffs that possess high antioxidant capacities have been an important focus of studies in analytical chemistry in recent years. Among the studied issues is the development of methodologies for chemically characterizing such plants and foods [1–3], the identification of compounds responsible for the antioxidant properties [4–6], and the proper measurement of the antioxidant power of specific components in the matrix [7–9]. For the characterization of antioxidant compounds, liquid chromatography coupled to mass spectrometry (LC–MS) has excelled as an analytical tool [1–3,5–7,10–12] because it is able to provide the high selectivity required for analyzing complex matrices, such as vegetable matrices. Moreover, the use of ultra-high performance LC (UHPLC) allows an increase in the separation efficiency, while the analysis times can be considerably reduced. Additionally, tandem mass spectrometry using triple quadrupole analyzers is a powerful quantification tool [13]. In recent years, studies on the contents of antioxidant compounds have been reported for oils [10–15], wine [11], fruits [2,7], honey [1] and medicinal herbs [3–6,12], wherein most of the methodologies developed with LC–MS used electrospray (ESI) as the ion source. However, few works investigated the effects of ion suppression on the analysis of antioxidant compounds in complex matrices [14–16]. It is well known, however, that various matrix effects act on the ionization mechanism of ESI sources [17–22], which subjects the methodologies to the occurrence of ion suppression.

The effect of ion suppression is characterized by a decrease in the signal of the analyte in the matrix compared to the same analyte concentration in a medium merely composed of pure solvents. In addition to ion suppression, ion enhancement can also be observed. Beyond the detectability, ion suppression can also be responsible for deviations in the repeatability and accuracy of analytical methods [23]. Signal suppression, when very pronounced, can lead to a false negative result, or in the case of using an internal standard (IS), the unequal suppression of the analyte and IS can lead to false positive results.

There are different mechanisms proposed for explaining ion suppression in LC–MS. The most widespread theory suggests a competition between the matrix components and analytes during the evaporation of droplets formed in the electrospray so that only some compounds reach the droplet surface and pass to a gas phase [24]. Moreover, interferents can modify the viscosity or surface tension of droplets produced by the electrospray, which affects the ionization efficiency. Competition for available electric

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charges can also occur in complex matrices, which can justify the eventual differences in signals observed for samples and standard solutions. However, ion suppression should not be considered as only a matrix effect. It can even be caused by co-eluting analytes such that their peak areas may not correspond to the sum of their individual responses. This phenomenon also indicates that there is a practical concentration limit of compounds that can be ionized simultaneously [25]. Lastly, other causes independent from the matrix, such as the mobile phase composition [19,26–28] or presence of contaminants from the analysis system [23], can also contribute to ion suppression or enhancement.

Different forms of evaluation for ion suppression have been singly described in the literature. Mallet et al. [29] described the evaluation of the influence of mobile phase additives on the signal intensity for acidic and basic drugs. In this study, it was observed that an increase in the additive concentration in the mobile phase caused a decrease in the analyte signals in most cases. Remane et al. [30] reported a study on the influence of co-eluted drugs on the effects of ion suppression and ion enhancement. The influence of the matrix composition was studied by Chico et al. [31], who evaluated the effect of different sample preparation approaches on the analysis of tetracyclines in feed samples. Herein, qualitative and quantitative assays were performed and showed suppression effects near 70% and enhancement effects of approximately 20% compared to the analysis of standard solutions. Recently, Mirnaghi et al. [11] performed a study to evaluate the analyte responses when subjected to different sample dilutions. This study aimed to measure the ion suppression caused by the competition of electric charges during the ionization process. Lastly, Avery [32] studied the differential ion suppression that can occur due to the wrong choice of an IS. This study compared the variation in the ratio of the analyte/IS areas in a series of samples of the same analytical matrix. In addition, the author compared the results with a commercial control of the sample, where the deviations were evaluated and the best IS was defined for the analytical method.

In addition to the quantification performed through the internal calibration, other methods of quantification are not free from ion suppression. The external calibration probably is the most susceptible method to ion suppression, due to the large difference in composition between standards and samples. A widespread calibration method is the matrix-matched calibration. This method requires a blank sample on which the external calibration curve must be built. Although this is a method much closer to reality than external calibration, it does not guarantee that all the samples to be analyzed present a similar response to electrospray ionization. Stüber and Reemtsma published a study which showed that none of the three aforementioned calibration methodologies are able to reduce the effects of ion suppression for samples with highly variable matrix [33]. In this study, they assumed that the calibration by standard addition provides the true analyte concentrations. Other authors have also reported the method of standard addition as the ideal calibration method to correct the effects of ion suppression [34], since the calibration curve is built on each of the samples to be analyzed. Unfortunately, this requirement makes it an extremely laborious method.

An alternative that is becoming more popular is the use of stable-isotope-labeled internal standards (SIL-IS) in quantitative methods. In this approach, stable substances with the same molecular structure of the analytes, but with deuterated sites or ^{13}C-labeled, are used as IS. Thus, co-elution of these internal standards with the analytes is guaranteed. Ideally, for each analyte should be used as IS its labeled analog, avoiding that SIL-IS demonstrate differential ion suppression. Each analyte is therefore ionized under the same elution conditions of the respective SIL-IS, correcting the effects of ion suppression. However, it is well known that such labeled standards are not easily found commercially and, when available, are extremely costly.

The aim of this work was to perform a complete evaluation of the different possible causes of ion suppression in the analysis of medicinal plant extracts by UHPLC-MS/MS using electrospray ionization, which is the main ion source used to analyze phenolic compounds. The comprehensive study of ion suppression in the determination of 24 phenolic compounds was carried out regarding the effects of mobile phase additives, the co-elution of compounds, the composition of the matrix, and the use of an IS (2-naphthol). The ion suppression effects were studied in 6 different extracts of medicinal plants from the Brazilian Amazonian region: *Mansoa alliacea*, *Bauhinia variegata* var. *variegata*, *Bauhinia variegata* var. *alboflava*, *Connarus perrottetii* var. *angustifolius*, *Cecropia obtusa* and *Cecropia palmata*. The studied compounds belong to the most frequently found phenolic classes in medicinal plants (flavonoids, flavones, hydroxycinnamic acids, coumarins, catechins and stilbenes), which have been analyzed as chemical and bioactive markers in extracts.

2. Experimental

2.1. Chemicals and reagents

The standards (+)-catechin, 2-naphthol, 3-acetyl coumarin, 3,6-dihydroxyflavone, 4-hydroxycoumarin, 6-hydroxycoumarin, apigenin, chlorogenic acid, chrysin, fisetin, galangin, gallic acid, kaempferol, luteolin, myricetin, *p*-coumaric acid, quercetin, quercitrin, resveratrol, rosmarinic acid, rutin, *trans*-cinnamic acid, and vanillic acid were obtained from Sigma–Aldrich (St. Louis, USA). Caffeic and ferulic acids were obtained from Fluka Analytical (Buchs, Switzerland). All the standards were of analytical grade with a minimum of 95% purity and were used as received.

Ultrapure water was obtained from a Milli-Q Synergy UV (Merck Millipore, Darmstadt, Germany) system. Methanol and acetonitrile (LC–MS grade) were obtained from Panreac (Castellar del Vallès, Spain). Acetic acid, formic acid, ammonium acetate, ammonium formate and ammonium hydroxide were obtained from Sigma–Aldrich (St. Louis, USA). Standard stock solutions (1000 mg L^{-1}) were prepared by dissolving appropriate amounts of substances in LC–MS grade methanol. All the solutions were stored in ambar glass recipients at −30 °C until their use. Working solutions of the studied phenolic compounds were prepared by dilution of the stock solutions in the respective solvents according to the optimization experiment performed.

2.2. UHPLC-ESI–MS/MS conditions

Separations were carried out on a UHPLC 1260 Infinity Binary system (Agilent, Santa Clara, USA), which was able to operate at pressures up to 600 bar using a reverse-phase Zorbax SB-C18 Rapid Resolution HD column (2.1 × 50 mm, 1.8 μm, Agilent) maintained at 40 °C. The injection volume was 5 μL, and the injected aliquots were acidified to a final concentration of 0.1% (v/v) acetic acid. The mixture of phenolic compounds was separated by using a gradient elution composed of 0.1% acetic acid (A) and acetonitrile (B) as the mobile phase at a constant flow rate (800 μL min^{-1}) according to the following elution program: 8.0% B (0.00–0.10 min); 8.0–25.8% B (0.10–3.45 min); 25.8–54.0% B (3.45–6.90 min); 54.0–100.0% B (6.90–7.00 min); and 100.0% B (7.00–9.00 min). In all the chromatographic separations, 2-naphthol was used as the IS.

The chromatographic effluent generated until 7.0 min into the run was ionized in an electrospray ionization source (ESI), which was optimized to give the best response for the analytes, especially for intensity to give signal stability. The optimized parameters for