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Direct coupling of supercritical fluid chromatography with tandem mass spectrometry for the analysis of amino acids and related compounds: Comparing electrospray ionization and atmospheric pressure chemical ionization

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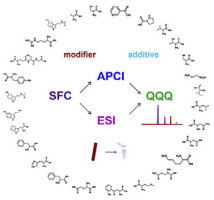
HIGHLIGHTS

- Direct coupling of supercritical fluid chromatography (SFC) with MS/MS.
- Comparison of the ionization performance using APCI or ESI.
- Separation of amino acids and other relevant metabolites.

GRAPHICAL ABSTRACT



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ABSTRACT

For acceptance of supercritical fluid chromatography (SFC) as a routine analysis method, the hyphenation to mass spectrometry (MS), which is typically achieved either by a splitting device or by the employment of an additional make up flow, has to be improved. Direct coupling of SFC to MS (/MS) would simplify the handling of this method. Consequently, this work focused on the direct coupling of SFC to mass spectrometry and the influence of the employed ion source on signal intensities of polar and ionic compounds in biological samples. A method for separating metabolites of the tryptophan pathway as well as other amino acids is shown. Results demonstrate that SFC is capable of separating analytes of polar and ionic nature. Modifications of the SFC system by cryostat cooling lead to higher temperature stability in the booster pump and therefore to a better reproducibility of retention times and a low dispersion nozzle inside the active back pressure regulator (ABPR) significantly improves peak shape and sensitivity when using the MS. The comparison of the ionization efficiencies using electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) in positive and negative ion mode shows analyte depending sensitivities. However, results indicate that APCI is better suitable for the ionization of amino acids with polar side chains, whereas ESI proved superior for the ionization of amino acids featuring hydrophobic residues. Analyte signals were suppressed with ESI when using a complex matrix such as human serum, but rather enhanced when using APCI.

1. Introduction

The development of sensitive, fast and reliable methods for the analysis of metabolites is urgently sought after for several applications, especially in the fields of clinical diagnosis, cancer research

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and fundamental research. Metabolites derived from tryptophan (Trp) like serotonin, N-acetyl-serotonin (NAS) and melatonin (Mel), as well as amino acids are involved in several biological pathways. The analysis of tryptophan, serotonin, N-acetyl-serotonin and melatonin with SFC-UV or LC-MS/MS was previously described [1–5]. Several publications deal with the analysis of free amino acids in various matrices with LC-MS/MS employing ion pairing chromatography [6–8] and hydrophilic interaction liquid chromatography (HILIC) [9]. However, the ionization efficiency for free amino acids employing SFC-APCI-MS/MS and SFC-ESI-MS/MS was not studied previously.

For the analysis of polar and ionizable compounds by SFC the addition of mainly alcoholic modifiers to carbon dioxide is necessary. Similar to HPLC, buffer salts or acids can be added to the modifier to improve peak shape or selectivity. Principally, all stationary phases used in HPLC can as well be used in SFC. Typically polar stationary phases are employed in SFC similar to normal phase chromatography or HILIC chromatography. The hyphenation of SFC with mass spectrometry is commonly achieved by splitting the flow and/or adding a make-up flow [10]. A make-up flow is usually employed to enhance ionization efficiency due to the addition of acidified organic solvent like methanol, especially when electrospray ionization is used. The quantification of metabolites without isotope-labelled internal standards in gradient mode can be challenging when splitting the flow due to the changing split-ratios. Direct coupling of the SFC with MS seems to be a convenient and promising alternative hyphenation. Nowadays, electrospray ionization is predominantly used in hyphenated systems. However, this ionization technique is concentration dependent, which means that signal intensity will be compromised when using high flow rates or higher modifier content in SFC. Furthermore, the ionization efficiency can be significantly influenced by the matrix of a biological sample resulting in ion suppression. An alternative ionization technique to ESI is atmospheric pressure chemical ionization [11]. APCI is a mass dependent ionization technique where ionization occurs in the gas phase and sensitivity is usually not as much influenced by matrix as in ESI. Studies which deal with the comparison of the ionization efficiency using SFC-APCI-MS/MS and SFC-ESI-MS/MS for the analysis of very polar compounds are limited [11–16]. It has been reported in the literature that small ionic or very polar analytes ionize better at high flow rates when using LC-APCI-MS [17,18]. The reverse applies in case of ESI.

The aim of this study was to evaluate the efficiency of direct coupling of SFC with MS/MS and to compare the ionization performance depending on the employed ion source APCI or ESI for the analysis of metabolites and amino acids in positive and negative ion mode. Investigations were done to evaluate the influence of modifiers of various polarities and additives such as ammonium formate (NH_4FA) and ammonium acetate (NH_4OAc) as well as acidified mobile phase conditions on separation and on ionization for both ion sources. Human serum was employed to evaluate the matrix effect and to compare the signal response of SFC-ESI-MS/MS and SFC-APCI-MS/MS in biological matrix.

2. Experimental

2.1. Materials

Methanol (MeOH, UHPLC-MS grade), water (H_2O, UHPLC-MS grade), ammonium acetate (NH_4OAc), formic acid (FA), and ammonium formate (NH_4FA) were purchased from Sigma Aldrich (Vienna). Acetic acid (AcOH) was bought from Carl Roth. Acetonitrile (ACN) was obtained from VWR and ethanol (EtOH) from AustrAlco. Serotonin was purchased from Alfa Aesar (Karlsruhe, Germany), melatonin (Mel) from TCI (Tokyo, Japan). N-acetyl-serotonin (NAS) and niacin were obtained from Sigma Aldrich (Vienna), whereas tryptophan was from Fluka (Vienna). The other biogenic amino acids were available in house due to other projects. Carbon dioxide (99.995%) was obtained from Messer (Austria).

2.2. Instrumentation and parameters

The SFC analyses were carried out using the Agilent 1260 SFC System directly coupled to an atmospheric pressure chemical ionization (APCI) source or to an electrospray ionization (ESI) source followed by a 6490 triple quadrupole mass spectrometer (QqQ) from Agilent Technologies.

For the separation of metabolites the Phenomenex Luna HILIC column (150 × 3 mm, 3 μm) was used. Gradient mode was employed using $scCO_2$ and an organic modifier with additives. As modifier MeOH, EtOH or a 1:1 mixture of MeOH:ACN was used and for better separation and ionization ammonium formate or ammonium acetate was employed as additive. The gradient started at 15% modifier, increased to 30% in 3 min, afterwards to 40% until 5 min, than to 45% until 6 min, keeping it constant for 1 min for washing and flushing back to starting conditions with a total run time of 8.5 min. The back pressure (ABPR) was set to 150 bar and the column temperature to 40 °C. The total system flow was 2 mL min^{-1}.

The following optimized parameters were used for the APCI ion source. The gas temperature was set to 200 °C, the APCI heater temperature to 350 °C, the capillary voltage to 4500 V, the nebulizer pressure to 20 psi and the gas flow to 14 L min^{-1} and the corona current was 4 μA. The following parameters were used when the electrospray ion source was employed. The gas temperature was set to 250 °C, the gas flow to 11 L min^{-1}, the Nebulizer pressure to 35 psi, the sheath gas heater temperature to 400 °C, the sheath gas flow to 12 L min^{-1} and the capillary voltage to 4000 V.

For comparison of the ion source performance amino acids, as well as metabolites derived from the tryptophan pathway, were employed namely serotonin, N-acetyl-serotonin, melatonin, niacin, and kynurenine. The MRM transitions and collision energies (CE) were optimized for positive and negative ion mode (Table 1).

2.3. Standards and samples

Stock solutions of 1 mg mL^{-1} dissolved in 0.1 M hydrochloric acid (HCl) for the investigated analytes were prepared and stored at −20 °C. For the modifier and additive study the stock solutions of each analyte were diluted with MeOH in order to have a final concentration of 10 ng μL^{-1}. Furthermore, a mix standard solution of all analytes (10 ng μL^{-1}), a mix standard of the metabolites derived from the tryptophan pathway (20 pg μL^{-1}) and a mix standard of melatonin, N-acetyl-serotonin, niacin, phenylalanine, threonine, tryptophan, methionine, glutamine and glutamic acid for spiking experiments (100 ng μL^{-1}) were prepared.

Human serum of different donors was pooled and used for evaluating the influence of the biological matrix on signal response employing APCI or ESI. Serum was chosen as there is no limitation in sample amount and as human serum presents one of the most complex biological matrices. 400 μL of MeOH were added to 200 μL serum for protein precipitation. The samples were vortexed, shaken on a thermo-shaker for 30 min at 25 °C and centrifuged for 15 min. An aliquot of 200 μL was transferred into HPLC vials equipped with glass micro inserts. For the serum blank samples 50 μL MeOH were added and for the spiked serum samples 25 μL of the spiking standard mixture as well as 25 μL MeOH were added in order to obtain a final concentration of 10 ng μL^{-1}.