Expanding the Analytical Design Space for Oxysterol Separation by HPLC: A Comparative Study Between Cyanopropyl and Octadecyl Stationary Phases

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Hyphenated mass spectrometric techniques (GC-MS and LC-MS) are considered as the tools of choice for the analytical characterization of oxysterols (OS) [1]. OS are oxidized cholesterol derivatives of great clinical interest due to their wide range of biological properties [2].
OS derivatization (*i.e.*, silylation) is required to increase OS volatility prior to the GC-MS analysis. On the other hand, OS derivatization is not a strict requirement for LC-MS methods, although it has been widely exploited to enhance the OS ionization yield [1,3]. Nonetheless, the use of derivatization procedures introduces new sources of variability that need to be thoroughly monitored. Thus, the use of derivatization-free LC-MS methods can significantly reduce the complexity of the pre-analytical steps.
The development of robust separation approaches is crucial to overcome the limits of MS detection. The latter are due to the formation of isomeric ions from non-isomeric OS owing to extensive in-source fragmentation processes that are triggered by electrospray (ESI) and atmospheric pressure chemical ionization (APCI). Moreover, the exploration of the separation capabilities of less commonly adopted stationary phases is crucial to reach the best compromise in terms of separation efforts.
In this study, the chromatography-based analytical Design Space modelling (DryLab) was employed to explore, optimize, and compare the performance of the cyanopropyl stationary phase (ES-CN) against the most commonly used octadecyl (C18) stationary phase. Multidimensional Design-of-Experiments (DoEs) were conducted to assess the effects of key separation parameters, including gradient steepness, column temperature, and mobile phase composition. The resulting visualized modelling maps captured the unique separation patterns of each stationary phase, revealing selectivity options and enabling the clear identification of optimal separation conditions. Individual DSs were systematically compared to define a common Method Operable Design Region (MODR), outlining shared conditions that achieve baseline separation of oxysterols across the two columns. This highlights the practicality of the methodology in facilitating column interchangeability studies and identifying operational conditions that enhance flexibility in both method development and routine analysis.

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