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Characterization of astaxanthin-enriched extracts from *Haematococcus pluvialis* by comprehensive two-dimensional liquid chromatography coupled to mass spectrometry detection

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The green microalga *Haematococcus pluvialis* have been widely studied due to its capacity of accumulate, under stress conditions, great amounts of astaxanthin, a high value carotenoid with biological properties. In the present work, two green processes, pressurized liquid extraction (PLE) and supercritical antisolvent fractionation (SAF) were integrated to obtain an astaxanthin-enriched extract from this microalga. PLE was carried out using ethanol as extracting solvent, for 20 min, at 100 bar; four different temperatures were studied in order to select the optimum conditions. Subsequently, the obtained extract was processed by SAF in order to further purify the carotenoids present. The SAF process was optimized using three experimental variables: (i) CO_2 pressure (100-300 MPa), (ii) percentage of water in the PLE extract (20-50%) and (iii) PLE extract/SC-CO_2 flow ratio (0.0125-0.05) The content of total carotenoids was evaluated in both extracts and raffinates. The higher carotenoid content in PLE was obtained at 50ºC. Then, optimum conditions for the enrichment in astaxanthin (using SAF) were at 300 bar, 0.05 feed/SC-CO2mass flow rate and 20 % (v/v) of water in the feed solution, achieving values of 120.3 mg carotenoids/g extract, which is significantly higher than the value obtained in PLE extract (91.6 mg carotenoids/g extract).

In parallel, the analytical characterization of the extracts was carried out. Due to the great amount of astaxanthin mono- and diesters present, the characterization of these samples is very challenging as a complex profile is obtained. For this reason, comprehensive two-dimensional liquid chromatography (LC×LC) was selected to carry out this task. A new fast LC×LC was optimized in order to get the full carotenoids profile of these extracts in less than 30 min. An amino column was employed in the first dimension (^{1}D) under isocratic conditions, whereas a short partially porous C30 column was selected for the fast second dimension (^{2}D) analyses. This is the first time that the use of this latter column is reported in an on-line LC×LC system. Combining the information from the DAD and MS detectors employed, different free and esterified carotenoids were identified in these samples.