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Continuous multicomponent quantification during supercritical fluid extraction applied to microalgae using in-line UV/Vis absorption spectroscopy and on-line evaporative light scattering detection

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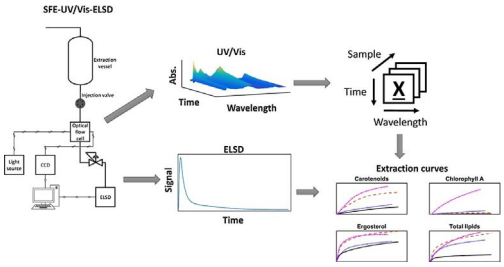
GRAPHICAL ABSTRACT



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ABSTRACT

A quantitative methodology based on in-line UV/Vis absorption spectroscopy and on-line evaporative light scattering detection for supercritical fluid extraction is proposed. The method was applied to the extraction of carotenoids, chlorophyll A, ergosterol and total lipids from microalgae. One regression technique and two curve resolution techniques were applied on the absorption spectroscopy data and evaluated, namely classical least squares, multivariate curve resolution by alternating least squares and parallel factor analysis (PARAFAC2). The two former both generated useful models, furthermore multivariate curve resolution also successfully enabled estimation of both spectra and concentration profiles of the analytes. The integrated extraction profiles of each analyte were compared with analysis of the collected fractions using reference analysis methods Precision, in regards to quantification of the analytes in the eluent, was better using in-line measurements compared to off-line measurements by UV/Vis absorption spectroscopy, supercritical fluid chromatography with mass spectrometry and liquid chromatography with UV/Vis detection.

1. Introduction

Supercritical fluid extraction (SFE) is a versatile technique for extracting lipophilic compounds from solid matrices. Carbon dioxide in combination with co-solvents is most commonly used as extraction phase in SFE. Due to the nature of the supercritical carbon dioxide ($scCO_2$), it offers low viscosities, high diffusivities along with tunable solubility by adjusting pressure, temperature and co-solvent composition [1]. Therefore, the extraction technique is rapid and it is also tweakable in terms of selectivity. Additionally, the technique uses none

Abbreviations: BPR, back-pressure regulator; CLS, classical least squares; ELSD, evaporative light scattering detector; EFA, evolving factor analysis; MCR-ALS, multivariate curve resolution by alternating least squares; NRMSE, mean normalized root mean square error; PARAFAC, parallel factor analysis; PCA, principal component analysis; $scCO_2$, supercritical carbon dioxide; SFE, supercritical fluid extraction; UHPSFC-DAD-MS, ultra-high performance supercritical fluid chromatography-diode array detection-mass spectrometry

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or minimal amount of organic solvents, thereby lowering the environmental impact and operational costs of both purchasing and disposing solvents.

Herrero et al. [2] point out SFE as an efficient and well-suited method for acquiring natural bioactive compounds from e.g. plants and algae. Carotenoids, chlorophyll, polyunsaturated fatty acids and polyphenols are examples of compounds of interest which can be extracted from microalgae, giving extracts with anti-oxidative and anti-inflammatory activity [2]. Much attention has also been given to large scale implementation of SFE of compounds from microalgae, due to mild extraction conditions and the absence of a subsequent solvent evaporation step [3–5]. However, it is not trivial choosing the appropriate extraction conditions and certainly the many possible process parameters and factors such as pressure, temperature, amount of co-solvent, flow rate, extraction time and particle size, also reinforces the difficulties to determine the optimum conditions.

The optimum extraction conditions for a given matrix and instrumental setup is usually determined empirically through various types of experimental designs [6]. However, by gaining a fundamental understanding of the SFE process, these factors could theoretically be predicted and optimum conditions could be established for extraction processes of various scales [7,8]. The study of extraction processes from a fundamental point of view generally requires many experiments in order to properly determine the underlying phenomena [9]. Since extraction curves are usually used for calibrating mechanistic models [10], these should preferably also have a high temporal resolution, i.e., a high measurement frequency in order to capture any occurring event. On-line detection hyphenated with SFE therefore offers efficient means to that particular end.

SFE has previously been coupled with both separation techniques such as chromatography and with in-line and on-line spectroscopic methods [11]. In-line detection has mainly focused on IR spectroscopy, e.g. to determine oil in water [12], petroleum in soil [13] or to study solubility [14]. Tena and Valcárcel [15] utilized a diode array detector to continuously monitor the extraction of caffeine from roasted coffee.

The before-mentioned studies did not involve the quantification of several analytes or did not have any present interferents. This is typically the case as several analytes with overlapping spectra are often being extracted simultaneously. Simultaneous quantification of total hops and water [16] and quantification of squalene with oil as interferent [17] have previously been performed with near-IR measurements followed by partial least squares (PLS) regression. However, PLS does not provide estimates of the unknown spectra.

Here curve resolution techniques such as multivariate curve resolution by alternating least squares (MCR-ALS) and parallel factor analysis (PARAFAC and PARAFAC2) could be feasible approaches to both acquire pure spectra and concentration profiles of several unknown analytes [18]. Curve resolution refers to chemometric methods that attempt to estimate, usually unknown or partially unknown, concentrations and spectra of pure compounds in mixtures. Numerous methods have been proposed for particularly two-way data, but also for higher dimensional data. These techniques have been widely used to study other processes, e.g. dissolution rates of pharmaceuticals [19], wastewater treatment [20] and chromatography [21]. In-depth theoretical aspects of curve resolution techniques have already been thoroughly discussed elsewhere and is therefore not covered in this work [22–24].

The aim of this work is to evaluate and assemble a complete methodology to quantitatively study the SFE process of several analytes simultaneously and continuously. Compounds that absorb light in the UV/Vis region are measured by absorption spectroscopy and the total extracted lipids are measured by an evaporative light scattering detector (ELSD). Extraction kinetics can then be studied without the need for collecting the eluent or any off-line analysis. Through the curve resolution techniques, the spectra, and thus the identification, of the compounds can be acquired using only data from the in-line measurements. The method is applied to study SFE of carotenoids, chlorophyll A, ergosterol and total lipids in microalgae. To the best of our knowledge this is the first time where more than two analytes are studied using in-line or on-line detection coupled to SFE and the first time that curve resolution techniques is utilized for spectroscopic data of SFE. We also aspire to show how multivariate and multiway techniques can be used to easily acquire large quantity of data that can be useful for studying the extraction processes.

2. Material and methods

2.1. Chemicals and samples

Ethanol (99.7%, Solveco, Rosenberg, Sweden) was used as make-up flow in SFE. Methanol (Scharlau, Sentmenat, Spain) and ammonium formate (Sigma-Aldrich, St. Louis, MO) used in ultra-high performance supercritical fluid chromatography-diode array detection-mass spectrometry (UHPSFC-DAD-MS) were of liquid chromatography (LC)-MS grade. Acetic acid (Scharlau) was of analytical grade and acetonitrile (Scharlau) was of GC grade, both used for LC analysis. Ultrapure CO_2 was provided by Air Products (Amsterdam, Netherlands). Analytical standards of the carotenoids lutein and neoxanthin were purchased from DHI (approx. 1 mg/L in methanol, Hørsholm, Denmark) and β-carotene (97%, Fluka, Buchs, Switzerland) were used for quantification by UHPSFC. Ergosterol (Santa Cruz Biotechnology, Dallas, TX) was used as standard in LC experiments. β-carotene (97%, Fluka, Buchs, Switzerland), chlorophyll A (Sigma-Aldrich, Steinhem, Germany) and canola oil from the local grocery store were used as standards during the SFE experiments. Dry standards, i.e., β-carotene, chlorophyll A and ergosterol were prepared in stock solutions. Chlorophyll A and ergosterol were dissolved in ethanol and β-carotene was dissolved in cyclohexane. All the following dilutions were performed in ethanol. The concentration of the stock solutions were determined by absorption spectroscopy using the molar absorption coefficients of Davies [25], Lichtenthaler [26] and Dorfman [27], respectively. This procedure is commonly applied as these analytes are difficult to acquire in high purity and they are easily degradable [28]. These measurements were performed on a Cary 100 Bio UV/Vis spectrophotometer (Varian Inc., Walnut Creek, CA), which was also used for analyzing collected extraction eluent. The microalgae, *Chlorella* sp., was purchased from Simsrisalg (Simrishamn, Sweden) and *Scenedemus* sp. was a mixture of collected freshwater species.

2.2. Supercritical fluid extraction

The system consisted of two ISCO 260D syringe-pumps (Teledyne Isco, Thousand Oaks, CA) used for pumping liquid CO_2, two Waters 515 HPLC pumps (Milford, MA, USA) used for pumping ethanol, a HP 5890 gas-chromatography oven (Hewlett-Packard, Wilmington, DE) functioning as oven, a Tescom 26-1700 back-pressure regulator (BPR) (Tescom Europe, Selmsdorf, Germany) and an Eltherm ELTC/3 thermoregulator (Eltherm Elektrowärmetechnik GmbH, Burbach, Germany) was used for heating the lining between the BPR and the ELSD model Sedex 55 (SEDERE, Alfortville, France). The absorption spectroscopy detection was performed using a flow cell with a 10 mm optical pathway (Knauer, Berlin, Germany), connected with optical fibers to a AvaLight DHc (deuterium and halogen) light source and an Avaspec 2048L charge coupled device (CCD) detector (Avantes, Eerbeek, The Netherlands). A Rheodyne injection valve with a 0.65 mL loop was used to introduce standards of known concentrations for calibration of the detectors. The liquid CO_2 pump was cooled by a Neslab RTE7 cooling bath controlled by a Digital One thermoregulator (Thermo Fisher Scientific, Waltham, MA). The whole setup is illustrated in Fig. 1. Definitions of on-line and in-line are adopted from Koch [29], where the process includes units between the pumps and the BPR. Thus the absorption spectroscopic detection is in-line because it is placed before the