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Technical Report

Comprehensive Two-dimensional Liquid Chromatography/Triple Quadrupole Mass Spectrometry: the Perfect Marriage

Enhanced Resolution and Sensitivity for a Challenging Food Case-Study—Red Chili Pepper Extract—

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Abstract:

This novel system combines the separation capabilities of comprehensive two-dimensional liquid chromatography (LC×LC), and the specificity and sensitivity of triple quadrupole mass detection (MSMS). The hyphenation of the techniques generates a powerful analytical system, capable of extremely high-resolution power, as well as targeted and untargeted analysis, simultaneously. The so-called selected reaction monitoring (SRM) mode in fact enhanced selectivity, reducing sample consumption and the need for tedious clean-up procedures, specifically for beta-carotene quantification in a red chili pepper extract.

Keywords: comprehensive LC×LC/MSMS, selected reaction monitoring, target analysis, carotenoids, red chili pepper extract

1. Introduction

The high complexity of many food samples places a great demand in terms of both separation capabilities, and specificity of detection. As far as separation is concerned, the implementation of multidimensional liquid chromatography (MDLC) techniques has provided enhanced resolving power for highly complex samples, especially in the "comprehensive" mode (LC×LC), in which the whole effluent from the first chromatographic dimension (D1) is transferred to a second chromatographic dimension (D2). As far as operation mode is concerned, "continuous on-line" techniques bring in additional advantages, including no need for flow interruption, no increase in overall analysis time, and capability of full automation, over other instrumental arrangements of two-dimensional LC, e.g. involving off-line transfer between the two dimensions (LC-LC), or the "stop-flow" techniques.

The enormous analytical advantages to be gained by coupling LC×LC separation to MS detection would be clear, if we consider the limitations of the two techniques when considered individually, and in which way a combination of the two have overcome them, generating the most powerful analytical tool today for non-volatile analytes. With respect to conventional LC-MS, the combination of two LC separations enhances physical separation of the components, reducing undesirable matrix effects arising from co-elutions. Maximizing the resolution is beneficial for subsequent MS detection, in terms of sensitivity and dynamic range, since it alleviates ion suppression effects due to insufficient separation, which may cause high abundant species to obscure the detection of less abundant ones.

Fig. 1 LC×LC/PDA/MSMS instrumentation

Unlike the UV detector, MS systems can also be employed with non-absorbing analytes, and can be operated in the full scan mode (TIC) or, more specifically, in tandem MS (MS-MS) experiments or in the selected ion monitoring (SIM) mode. Constant neutral loss or precursor ion scanning techniques help in distinguishing the ions of interest from unspecific matrix components simply by monitoring only those *m/z* values which originate from a characteristic fragmentation pattern. The so-called selected reaction monitoring (SRM) mode enhances selectivity and lowers detection limits, therefore reducing sample consumption; additionally, the SRM approach can also decrease analysis times by reducing the need for clean-up procedures.

This technical report describes a novel LC×LC/PDA/MSMS instrument, capable of extremely high-resolution power, as well as targeted and untargeted analysis, simultaneously (Fig. 1).

The system was successfully employed for the characterization of the native carotenoids in red chili pepper (Fig. 2), also allowing for quantification of *beta*-carotene, at sub-ppm level.

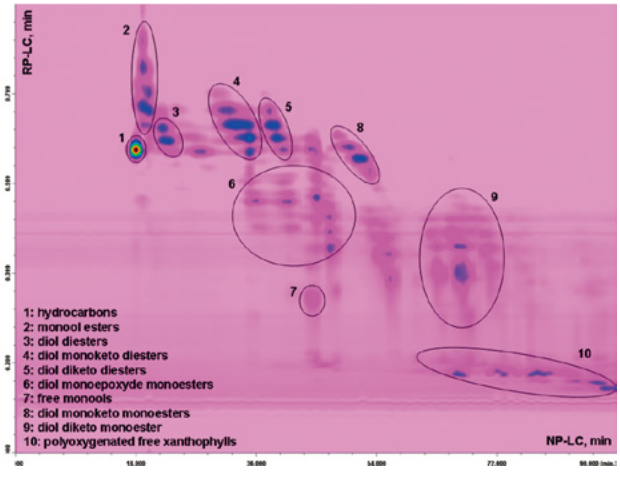

Fig. 2 NP-LC×RP-LC Plot of a red chili pepper extract

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2. Experimental

2-1. Instrument

- Shimadzu CBM-20A controller
- Shimadzu LC-30AD dual-plunger parallel-flow pumps (D1-LC)
- Shimadzu DGU-20A5R degassing unit (D1-LC)
- Shimadzu LC-30AD dual-plunger parallel-flow pumps (D2-LC)
- Shimadzu DGU-20A3R degassing unit (D2-LC)
- Shimadzu CTO-20AC column oven
- Shimadzu SIL-30AC autosampler
- Shimadzu SPD-M30A photo diode array detector (1 µL flow cell)
- Shimadzu LCMS-8030 (DUIS source)

For connecting the two dimensions: two electronically-controlled 2-position, 6-port high pressure switching valves FCV-32AH (with two 20 µL empty loops), Fig. 3 and Fig. 4.

2-2. Software

- Shimadzu LabSolutions (Version 5.60 SP2)

2-3. 2D Software

- ChromSquare (Version 2.0) from Chromaleont, Messina, Italy

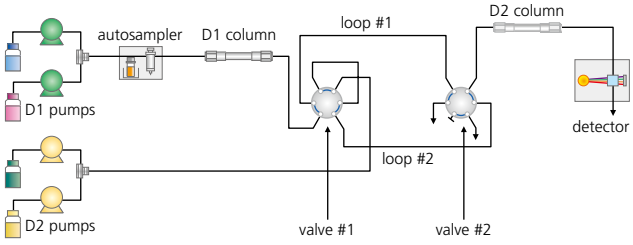

Fig. 3 Schematic of the 2D system and the switching valves

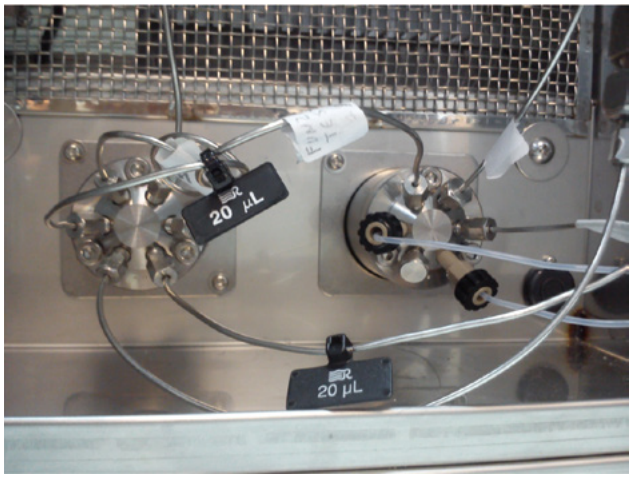

Fig. 4 Interior of the CTO-20AC oven with the switching valves and loops

2-4. Chromatographic Methods

First dimension (Normal-phase)	
Column	: Ascentis ES-Cyano, 250 mmL. × 1.0 mmI.D., 5 µm d.p. (Sigma-Aldrich/Supelco, Bellefonte, PA, USA)
Mobile phase	: (A) Hexane (B) Hexane/Butylacetate/Acetone (80/15/5, v/v/v)
Gradient	: 0–5 min, 0% B, 5–65 min, to 100% B
Flow rate	: 20 µL/min
Column oven	: 30 °C
Injection vol.	: 2 µL

Second dimension (Reversed-phase)	
Column	: Ascentis Express C18, 50 mmL. × 4.6 mmI.D., 2.7 µm d.p. (Sigma-Aldrich/Supelco, Bellefonte, PA, USA)
Mobile phase	: (A) Acetonitrile (B) Isopropanol
Gradient	: 0.01 min, 0% B, 0.01–0.17 min, to 50% B, 0.17–0.27 min, 50% B, 0.27–0.54 min, to 80% B, 0.54–0.93 min, 80% B, 0.94 min, to 30% B (Fig. 5).
Flow rate	: 4 mL/min
Column oven	: 30 °C
Modulation time	: 1 min
Loop size	: 20 µL

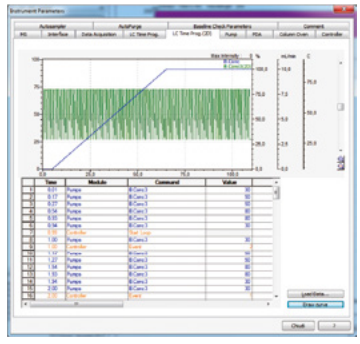

Fig. 5 Instrument parameters view of the LabSolutions software with D1 and D2 gradients

2-5. Detection

PDA range: 250–550 nm; sampling rate 12.5 Hz; time constant 0.080 sec

LCMS-8030: DUIS positive mode; from the LC system 800 µL/min of the D2 flow were directed to the probe. For the splitting device, a stainless steel microvolume connector was used (1/16", 0.15 mm bore), from VICI (Valco Instruments Co. Inc.).

Full scan mass spectral range: 410–1200 *m/z*; event time: 0.1 sec; nebulizing gas (N_2) flow: 2.5 L.min^{-1}; drying gas (N_2) flow: 20 L.min^{-1}; Heat block temperature: 400°C; desolvation line (DL) temperature: 250°C; Interface voltage: 4.5 kV. MS parameters were optimized for MRM of the following transitions: *m/z* 536.40 to *m/z* 444.30 (quantifier ion) and *m/z* 536.40 to *m/z* 105.00 (qualifier ion).

2-6. Sample Preparation

The red chili pepper (*Capsicum annuum* L.) sample was purchased in a local market. For the extraction of intact carotenoids (not saponified), 200 g of red chili pepper homogenate were treated with three consecutive 300-mL aliquots of a methanol/ethyl acetate/petroleum ether (1:1:1, v/v/v) mixture. The extracts combined were filtered through paper, evaporated to dryness under vacuum (30 °C), and the dry residue was then dissolved in 3 mL of a methanol/*tert*-butyl methyl ether (1:1, v/v) mixture and filtered through a 0.45 mm Acrodisc nylon membrane (Pall Life Sciences, Ann Arbor, MI, USA).

3. Results and Discussion

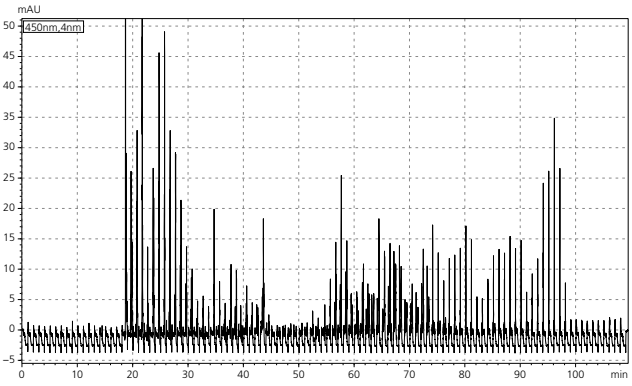

Fig. 6 Postrun window of the LabSolutions software showing the NP-LC×RP-LC chromatogram of red chili pepper

Fig. 7 Launching the ChromSquare software

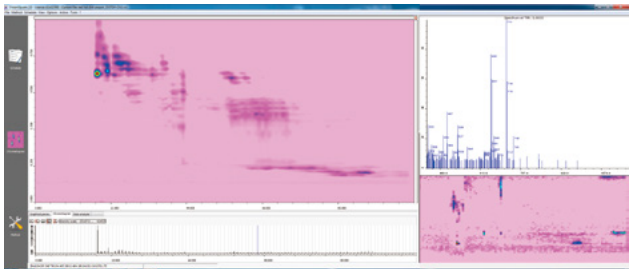

Fig. 8 Window of the ChromSquare software
Left: 2D plot of the MS data (full scan) and the raw chromatogram
Top-right: MS spectrum

Chromatography on the cyano stationary phase allowed a good separation of the carotenoids in groups of different polarity in the first dimension, as can be seen, with retention times increasing in the order: hydrocarbons < monool esters < diol diesters < diol monoketo diesters < diol diketo diesters < diol monoepoxide monoesters < free monools < diol monoketo monoesters < diol diketo monoesters < polyoxygenated free xanthophylls (Fig. 2). On the other hand, the C18 column allowed the separation of carotenoids within each class, according to their increasing hydrophobicity and decreasing polarity (for components of the same class, the elution order increases with the number of carbon atoms of the fatty acid chain).

Identification of the separated compounds was achieved by means of both PDA and MS detection (DUIS). The latter represents a powerful analysis tool for unknown molecules; especially in the case of carotenoids, operation of the interface under both positive and negative mode offers the double advantage of improved sensitivity and/or identification power. MS spectra obtained under negative ionisation mode are in fact dominated by the presence of very intense pseudomolecolar ions $[M]^-$, which make identification/quantitation of low-abundant components easier; on the other hand, abundant fragmentation is, generally, observed under positive ionization, and fragment ions can help in structure elucidation through dedicated software/database.

.....through dedicated software for 2D data handling

ChromSquare workstation software is designed for visualizing, processing, and reporting on data obtained by two-dimensional chromatography. Main features are:

In 2D plots, chromatographic points correspond to a pair of numerical values, i.e. time and intensity (absorbance).

In 2D chromatography the whole chromatogram is divided into a set of modulations, according to a modulation time; in this representation, the time value of the chromatographic point corresponds to two time values: the overall time value, represented along the x-axis, and the time value of the single modulation, represented along the y-axis.

The intensity value, or absorbance, corresponds to the third dimension; in the 3D representation (see Fig. 7) this corresponds to the z-axis, whereas in the 2D representation this corresponds to a color level; the whole set of all points generates a contour map.

.....to 2D plot for visualization and more.....

The 2D plot obtained from red chili pepper is shown in Fig. 8. It renders chemical patterns, in which the compounds are characteristically distributed according to increasing polarity (NP-LC separation, x-axis) and increasing hydrophobicity (RP-LC separation, y-axis).

For the quantitative data analysis, *beta*-carotene (*m/z* 536.40) transitions at *m/z* 444.30 (quantifier ion) and *m/z* 105.00 (qualifier ion) were selected for the construction of a calibration curve, in the 1 ppb–10 ppm concentration range. Very good linearity was observed, with regression line equation: y = 410259x + 49414; linear correlation coefficient (R) = 0.998976. The amount of *beta*-carotene was afterwards determined, in the real sample analyzed, as equal to 1.22 ppm. Fig. 9 shows an expansion of the 2D plot, with the *beta*-carotene blob and the MS spectrum with the two transitions.

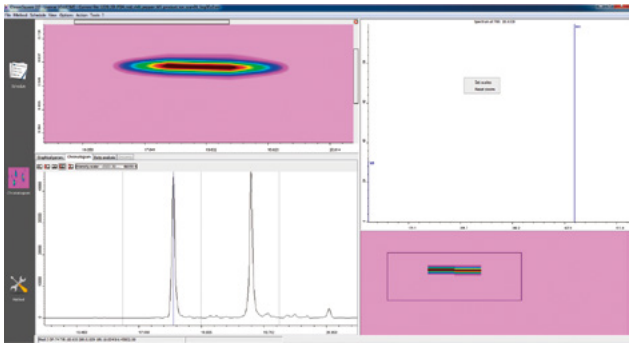

Fig. 9 Window of the ChromSquare software
Top left: 2D plot of the MS data showing *beta*-carotene at *m/z* 536.40
Bottom left: raw data of three consecutive modulation times (1.00 min each)
Top right: MS spectrum showing the transitions at *m/z* 444.30 (quantifier ion) and *m/z* 105.00 (qualifier ion)

4. Conclusions

The combination of a triple quadrupole mass spectrometer (LCMS-8030) to an LC×LC system generates an extremely powerful analytical system, capable of extremely high-resolution power, as well as targeted and untargeted analysis, simultaneously.

For the separation/identification/quantification of highly complex samples unique features of this instrumentation consist of: high resolution power and reproducibility, fast analysis time, full automation, high sensitivity and high mass stability, fragment information (MS/MS), and fast cycle time (including polarity switching).

Moreover, the front-end LC×LC separation renders 2D plots with chemically-similar compound patterns, which may be of great help in the identification of unknowns, in the absence of standard components or of a corresponding MS library spectrum.

The advantages to be gained by coupling 2D-LC to MS can be summarized as follows:

- handle complex samples
- reduce matrix complexity entering the probe
- reduce ion supression
- detect even low abundant signals
- get structural information
- increasing confidence in the result

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