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

Enhanced Resolution and Sensitivity for a Challenging Food Case-Study
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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 betacarotene quantification in a red chili pepper extract.

Keywords: Comprehensive LC×LC/MSMS, Selected reaction monitoring, Target analysis, Carotenoids

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 (MDLC) liquid chromatography 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 in 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.

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 (Figure 1).

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

Figure 1. LC×LC/PDA/MSMS instrumentation.

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

Instrument
- Shimadzu CBM-20A controller
- Shimadzu LC-30AD dual-plunger parallel-flow pumps (D1-LC)
- Shimadzu DGU-20A5R degasser (D1-LC)
- Shimadzu LC-30AD dual-plunger parallel-flow pumps (D2-LC)
- Shimadzu DGU-20A3R degasser (D2-LC)
- Shimadzu CTO-20AC column oven
- Shimadzu SIL-30AC autosampler
- Shimadzu SPD-M30A photo diode array detector (1.8 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), Figure 3 and Figure 4.

Software
- Shimadzu LabS heter (Version 5.60 SP2)

2D Software
- Chromsquare (Version 2.0) from Chromaleont, Messina, Italy

Chromatographic Methods

First dimension (Normal-phase)
Column: Ascentis ES-Cyano, 250x1.0 mm, 5 µm d.p.
(Sigma-Aldrich/Supelco, Bellefonte, PA, USA)
Mobile phase: (A) Hexane
(B) Hexane/Butyl acetate/Acetone (80/15/5, 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, 50x4.6 mm, 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 (Figure 5).
Flow rate: 4 mL/min
Column oven: 30 °C
Modulation time: 1 min
Loop size: 20 µL

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 (N₂) flow: 2.5 L.min⁻¹; drying gas (N₂) flow: 20 L.min⁻¹;
Heat block temperature: 400°C; desolvation line (DL) temperature: 250°C;
Interface voltage: 4.5 kV. For MRM optimization, beta-carotene transitions
at

Sample preparation
The red chili pepper sample (Capsicum annuum L.) 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-buty methyl ether (1:1, v/v) mixture and filtered
through a 0.45 mm Acrodisc nylon membrane (Pall Life Sciences, Ann
Arbor, MI, USA).
Results and Discussion

Figure 6. Postrun window of the LabSolution software showing the NP-LCxRP-LC chromatogram of red chili pepper.

From raw data file......

Chromatography on the cyanostationary 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 < mono-ol-esters < di-ol-di-esters < di-ol-mono-epoxide-di-esters < di-ol-mono-keto-di-esters < free-mono-ols < di-ol-mono-epoxide-mono-esters < di-ol-di-keto-mono-esters < di-ol-di-keto-di-esters < poly-oxygenated-free-xanthophylls.

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 pseudomolecular 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 Figure 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 for red chili pepper is shown in Figure 8. It renders chemical patterns, in which the compounds are characteristically distributed according to increasing polarity (NP-LC separation, x-axys) and increasing hydrophobicity (RP-LC separation, y-axys).

For the quantitative data analysis, a calibration curve was obtained for beta-carotene, in the 10 ppb-10 ppm range.

MS parameters were optimized for the selected reaction monitoring, using the transitions of m/z 536.30 to 444.30 (CE -18.0), as a quantifier ion, and to 105.00 (CE 54.0) as a qualifier ion (Figure 9).

Parameters from linear regression analysis were as follows: slope, 40701; intercept, 1716; correlation coefficient, 0.9998. Afterwards, the quantification of beta-carotene in red chili pepper was achieved, as 2 ppm.
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 in: high resolution power and reproducibility, fast analysis time, full automation, high sensitivity and high mass stability, fragment information (MS²), 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 are available, or of a corresponding MS library spectrum.

The advantages to be gained by coupling 2DLC to MS can be summarized as follows:

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

Figure 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 444.30 (quantifier ion) and 105.00 (qualifier ion).