SHIMADZU
Excellence in Science

PO-CON1230E

Ultra high pressure comprehensive two-dimensional liquid chromatography combined with hybrid mass spectrometry for the elucidation of carotenoids in red chili peppers

IMSC 2012 PWe-197

[1]Marcus Mreyen, [2,3]Francesco Cacciola,
[3,4]Paola Donato, [5]Daniele Giuffrida, [3]Germana Torre,
[3,4]Paola Dugo, [3,4]Luigi Mondello
[1]Shimadzu Europa GmbH, Duisburg, Germany;
[2]Chromaleont S.r.l., Messina, Italy;
[3]Dipartimento Farmaco-chimico, Messina, Italy;
[4]Centro Integrato di Ricera (C.I.R.), Roma, Italy;
[5]Dipartimento di Scienze degli Alimenti e dell´ Ambi,
Messina, Italy

SHIMADZU
Excellence in Science

Ultra high pressure comprehensive two-dimensional liquid chromatography combined with hybrid mass spectrometry for the elucidation of carotenoids in red chili peppers

Overview

A novel NP-LC×RP-UHPLC application is here presented, consisting of a micro-bore column for the first dimension (1D) separation, and two serially coupled C18 columns packed with fused-core particles in the second dimension (2D), operated under different gradient and modulation times. Performances of these two set ups were evaluated, in comparison to conventional NP-LC×RP-LC, in terms of peak capacity values (nc), under-sampling, and orthogonality effects, for carotenoid fingerprinting in red chili pepper by means of both PDA and LCMS-IT-TOF data.

Introduction

The extent to which mass spectrometry can be of help in unravelling post-column co-eluting components is witnessed by the increasing number of applications using MS as a third or higher added dimension to comprehensive LC×LC techniques, in which the entire analyte sample is subjected to the "2D advantage".

Materials and Methods

Samples

Red chili pepper (*Capsicum annuum L.*) was extracted with methanol / ethyl acetate / petroleum ether (1:1:1, *v/v/v*).

Instrumentation and software

Shimadzu Nexera LC-30A, coupled to an SPD-M20A photo diode array detector, and a LCMS-IT-TOF mass spectrometer. LC×LC interface: two high speed / high pressure two-position, 6-port switching valves (Fig. 1). Both dimensions and the valves were controlled by the LCMSsolution® software (Version 3.50.346, Shimadzu). The LC×LC data were visualized and elaborated using Chromsquare® ver. 1.5 software (Chromaleont, Messina, Italy).
PDA: 250-550 nm (12.5 Hz; 0.08 s); LCMS-IT-TOF: APCI (+/-), 200-1200 *m/z*, 400°C; detector, 1.50 kV; CDL, 250°C; block heater, 250°C; nebulizing gas flow (N2), 2.5 L/min; ion accumulation time, 30 ms; repeat, 3; ASC, 70%.

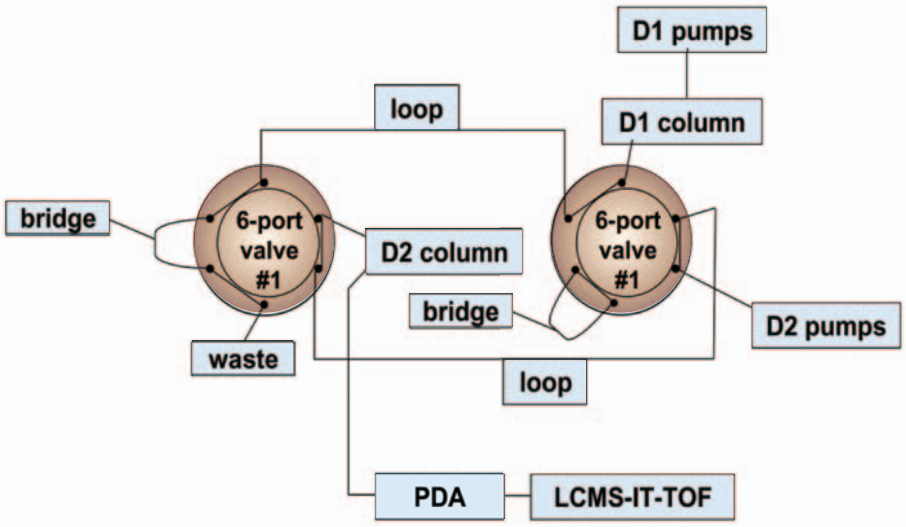

Fig. 1 Schematic view of the LC×LC system employed in this work.

LC×LC/MS
- Solves peak co-elutions
- Handles complex samples
- Detects low abundant signals
- Reduces ion supression
- Aids structural elucidation
- Allows more robust quantification
- Increases confidence in result

NP-LC×RP-LC
- Orthogonal separation modes
- High resolving power
- Fast analysis time
- Fully automated
- High reproducibility

LCMS-IT-TOF
- High mass accuracy
- Fragment information (MS^n)
- High mass resolution in all MS modes
- Good precursor ion selection
- Fast cycle time and polarity switching

Ultra high pressure comprehensive two-dimensional liquid chromatography combined with hybrid mass spectrometry for the elucidation of carotenoids in red chili peppers

NP-LC×RP-LC conditions (Set up #1)

[1]D: Ascentis ES Cyano, 250 × 1.0 mm I.D., 5 mm; Mobile phase: (A) *n*-hexane; (B) *n*-hexane/butyl-acetate/acetone (80:15:5, *v/v/v*). Gradient: 0-5 min, 100% A; 5-65 min, to 0% A; hold for 45 min. Flow rate: 10 µL/min. Oven: 30°C. Injection volume: 2 µL.

[2]D: Ascentis Express C18, 30 × 4.6 mm I.D., 2.7 mm; Mobile phase: (A) water/ACN (10:90, *v/v*); (B) IPA. Gradient: 0.01 min, 30% B; 0.12 min, to 50% B; hold for 0.08 min; 0.40 min, to 80% B; hold for 0.30 min; 0.71 min, to 30% B; hold for 0.04 min. Flow rate: 4 mL/min. Column oven: 65°C. Modulation time: 0.75 min.

NP-LC×RP-UHPLC conditions

[1]D: all conditions as in Set up #1. **[2]D**: Two Ascentis Express C18, 30 × 4.6 mm I.D., 2.7 mm serially connected; Mobile phase: (A) water/ACN (10:90, v/v); (B) IPA. Flow rate: 4 mL/min. Column oven: 65°C.

Set up #2. Modulation time: 1.50 min. Gradient: 0.01 min, 30% B; 0.25 min, to 50% B; hold for 0.15 min; 0.80 min, to 80% B; hold for 0.60 min; 1.41 min, to 30% B; hold for 0.09 min.

Set up #3. Modulation time: 1.00 min. Gradient: 0.01 min, 30% B; 0.17 min, to 50% B; hold for 0.10 min; 0.54 min, to 80% B; hold for 0.39 min; 0.94 min, to 30% B; hold for 0.06 min.

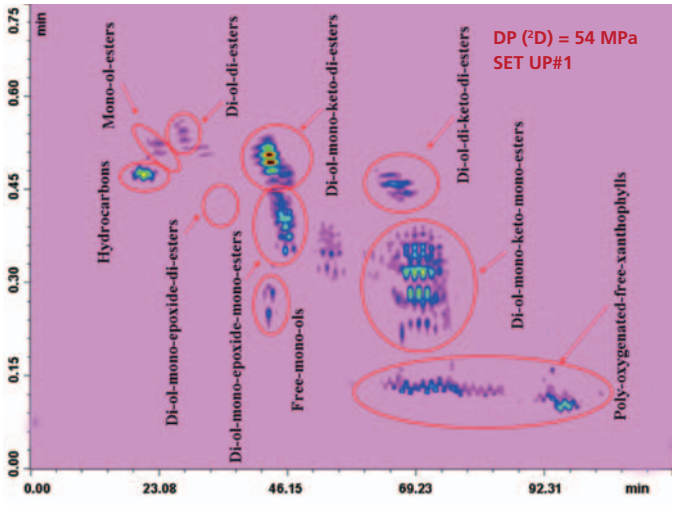

Fig. 2 NP-LC×RP-LC analysis of free carotenoids and esters in red chili pepper (modulation time: 0.75 min).

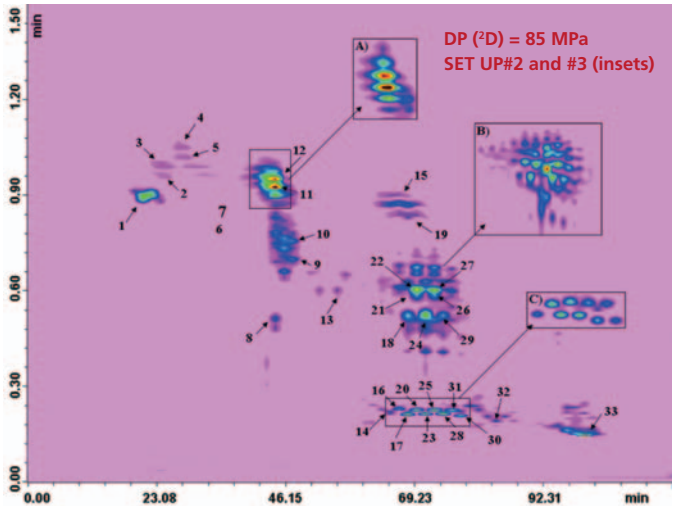

Fig. 3 NP-LC×RP-UHPLC analysis of free carotenoids and esters in red chili pepper (modulation time: 1.50 or 1.00 min, insets A,B,C).

Results and Discussion

Results obtained from the NP-LC×RP-LC analysis of free carotenoids and carotenoid esters in the red chili pepper extract are shown in the contour plot of Fig. 2 in the [1]D, the Cyano phase allowed a good separation of the carotenoids into 10 classes of different polarity (circles); in [2]D, carotenoids were separated on the C18 column according to their increasing hydrophobicity and decreasing polarity (for components of the same class, elution order increases with the number of carbon atoms of the FA chain). Results obtained from the NP-LC×RP-UHPLC analysis of the sample are shown in the contour plot of Fig. 3. The improvement in separation power is clear from a visual inspection of the [2]D plot, especially for the free-xanthophyll and the di-ol-mono-keto-mono-ester classes.

Ultra high pressure comprehensive two-dimensional liquid chromatography combined with hybrid mass spectrometry for the elucidation of carotenoids in red chili peppers

Further improvement of the NP-LC×RP-UHPLC system was attained by reducing the modulation time from 1.50 to 1.00 min with the stepwise gradient modified, accordingly. Better fractionation of the ^{1}D eluate improved the chromatographic separation, as it can be clearly seen from the insets in the ^{2}D plot (Fig. 3).

LCMS-IT-TOF allowed to discriminate between compounds showing nearly identical UV-absorption properties (same chromophores). An example is represented by the two diol-monoketo-diesters labelled as 11 and 12 in Fig. 3, Table 1, and Fig. 4 the absorption spectra of these two molecules in fact overlap, while the *m/z* ions allow to easily distinguish one from the other. Distinctive fragmentation obtained by MS/MS helps in elucidation of the structure: different fragment ions arise from the loss of fatty acid moieties. Up to 33 carotenoids, either in their free, or in the esterified form, were positively identified without the need for standard compounds.

Table 1 NP-LC×RP-UHPLC/PDA and LCMS-IT-TOF (APCI) carotenoid fingerprint in a red chili pepper extract.

No.	Compound	PDA (nm)	Molecular Formula [M]	$[M]^{-}$ calculated	$[M]^{-}$ observed	Error (ppm)	Fragment ions
11	Capsanthin-C12:0,C14:0	474	$C_{66}H_{104}O_5$	976.7884	976.7843	- 4.19	777, 749, 549
12	Capsanthin-C14:0,C14:0	474	$C_{68}H_{108}O_5$	1004.8197	1004.8152	- 4.48	777, 549

Table 2 Peak capacity, n_C of the set ups: Theoretical, Corrected (orthogonality), Effective (under-sampling).

		SET UP #1	SET UP #2	SET UP #3
2Dn_C	Theoretical	990	1395	1125
	Corrected	858	558	775
	Effective	727	377	984

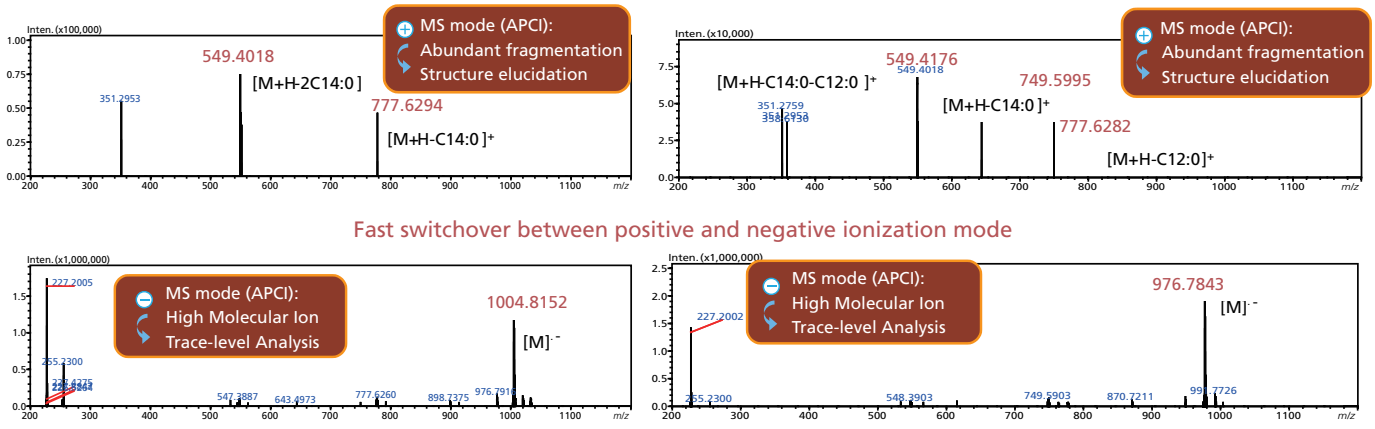

Fig. 4 Fast polarity switching allows both trace-level analysis and structure elucidation.

Conclusion

Performance evaluation of the three systems is shown in Table 2. The improved separation power and identification power of the LC×LC/MS allowed more confident structure elucidation and reliable quantification.

First Edition: September, 2012

Shimadzu Corporation
www.shimadzu.com/an/