Timed relay contact closure controlled system for parallel second dimensions in multi-dimensional liquid chromatography

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Abstract
Objective: Short-chain triacylglycerols (TAGs) in lipid extracts of biological samples are not sufficiently resolved using conventional reversed-phase separation on two C18 columns in series, or using a two-dimensional chromatographic separation with a silver ion column as the second dimension (2D). An additional dimension of separation was required.

Results: The hardware and software components to allow a second second-dimension (2D) separation and three total separation dimensions were developed. Two contact closure (CC) activated 4-port, 2-position valves (4P2PVs) for ultra-high performance liquid chromatography (UHPLC) were joined together and used for one of two second dimensions in comprehensive two-dimensional liquid chromatography (2D-LC) coupled to four mass spectrometers simultaneously in parallel in an LC1MS2 × (LC1MS1 + LC1MS1) = LC3MS4 configuration. A timed contact closure circuit (TCCC) controlled the two UHPLC valves, operated by repetitive CCs for the 4P2PVs. The TCCC-controlled 4P2PVs were used to direct a portion of the 1D eluent to one of the two 2D's for separation by a quaternary UHPLC system that was not allowed by the commercial 2D-LC system. The 1D separation was a non-aqueous reversed-phase HPLC instrument used for separation of TAGs; the commercial 2D-LC 2D binary UHPLC was used for silver-ion chromatography of unsaturated TAGs; and the CC-controlled second 2D was used for separation of short-chain (SC) saturated TAGs.

Keywords: 2D-LC–MS, APCI-MS, ESI-MS, APPI-MS, Contact closure

Introduction
Instruments for comprehensive two-dimensional liquid chromatography (2D-LC) are now routinely available. Comprehensive 2D-LC produces a separation on a first-dimension (1D) column and all or a portion of the effluent is directed to a second-dimension (2D) column, with every peak in the 1D transferred to the 2D column, in contrast to heart-cutting and other 2D-LC approaches. However, there are limitations inherent in commercially available 2D-LC instruments. For instance, the 2D-LC system was designed for the use of one 2D binary pump ultra high performance liquid chromatography (UHPLC) system coupled with the 1D quaternary pump HPLC (or UHPLC) system. The system could not utilize newer quaternary UHPLC systems for the second dimension, since among other things, the software only allowed two solvent channels to be configured for the 2D in 2D-LC.

More separation options were needed than were commercially available because milk triacylglycerols (TAGs) are very complex and could not be adequately separated using 1D-LC, due to the presence of numerous isobaric isomers. Also, milk TAGs contain a large number of very short-chain fatty acids (SCFAs), down to C4, that were not retained well on the conventional C18 columns normally used for TAG analysis. Furthermore, the SCFAs are saturated, so the TAGs that contained them were not separated using the silver-ion UHPLC used as the 2D to separate unsaturated TAGs, especially those containing trans double bonds [1, 2].

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To overcome these limitations, we employed two contact closure (CC) controlled 4-port, 2-position valves (4P2PVs) that were joined together to emulate the eight-port switching valve on the commercial 2D-LC system. To automate the CCs, the 4P2PVs were connected to a timed contact closure circuit (TCCC) that provided consistent timed CCs to switch the 4P2PVs uniformly throughout the chromatographic separation. Control of the TCCC was incorporated into the wireless communication contact closure system (WCCCS) that we previously reported [3]. Using this prototype instrument configuration we were able to perform separations employing three LC systems and four mass spectrometers (LC3MS4), in which the 1D HPLC separation was monitored using electrospray ionization mass spectrometry (ESI-MS) and atmospheric pressure chemical ionization (APCI) MS in parallel (= LC1MS2), and two 2D UHPLC separations were conducted simultaneously in parallel, with a binary UHPLC using a silver-ion column monitored using atmospheric pressure photoionization (APPI) MS (= LC1MS1) and a quaternary UHPLC using a C8 column monitored using ESI-MS (= LC1MS1). Thus, we report here the hardware and software components necessary to accomplish 2D-LC having two parallel second dimensions, with detection by four mass spectrometers simultaneously in parallel, plus 7 other detectors, for \( LC_{1MS1} \times (LC_{1MS1} + LC_{1MS1}) = LC_{3MS4} \). This new experimental arrangement provides a new tool to allow us to conduct method development for complex lipid analysis.

**Main Text**

**Materials and methods**

Two Cheminert nanovolume 4P2PVs with microelectronic actuator (#C85U-6674EMT, Valco Instruments Co., Inc., Houston, TX, USA) rated to 20,000 psi (1379 bar) were joined together as shown in Fig. 1a. The microactuators for the two valves were both connected to a 12 V 10A 10-bit DIP-switch-controlled Infitech binary digital power timed delay relay (#BRKR1A411, Infitech, Inc., Syracuse, NY, USA) having a time delay range of 0.1 to 102.3 s as shown in Fig. 1b. The 12 V power required to initiate the on/off recycling of the TCCC was wired through relay #14 of the previously reported WCCCS receiver boards (Ref. 3 Figures 2B, 3). Both WCCCS sender boards were rewired to add individual controls of relays B, C, and D, as shown in Fig. 2. Relay A was already wired through the single switch on the sender board mounting units, to provide a timed relay as a longer start signal. Switch #14 on the switch distribution manifold made the voltage to the TCCC from the WCCCS switchable between Relay B from the Agilent 1200’s G1329A autosampler or from the Agilent 1290 Infinity Flex II’s Universal Interface Box II (UIB II), either of which was controlled by the relay control timetable in respective versions of OpenLab ChemStation (OLCS) C.01.09 software running on both systems.

Repetitive CCs from the TCCC in Fig. 1b were connected to the microelectric actuator control modules (MACMs) shown in Fig. 1c. The CC connections to the two MACMs were joined together to ensure they activated simultaneously, and connected to the horizontal and one vertical spade terminal on the TCCC, as shown as in Fig. 1b. Because both connections were connected to one of the two vertical terminals, the time set on the DIP switch was one-half of the valve cycle time. The maximum setting of the model of TCCC demonstrated here was 102.3 s, so the TCCC could be used for cycle times up to 204.6 s (3.41 min). 2D separations had run times of 1.91 min (=114.6 s), so the DIP switch was set to 57.3 s, or binary 100011101.

The same mass spectrometers and other detectors that were used for the previous reports on LC2MS4 were used for the 1D and 2D(1) [2, 4]. Detailed descriptions of the instruments and parameters used for comprehensive 2D-LC with quadrupole parallel MS (LC2MS4 = LC1MS2 × LC1MS2) were given previously (see Supplemental Materials to Ref. 4). The Agilent Infinity Flex II quaternary UHPLC has been added since that report, and is used for the 2D(2) separation. The exact same parameters that were used previously were used for the UV and fluorescence (FLD) detectors on the new UHPLC system [4]. The evaporative light scattering detector (ELSD) (G4261B, Agilent Technologies, Santa Clara, CA, USA) was moved from the monitoring the 1D to monitoring the 2D(2). The overall arrangement of all liquid chromatographs, auxiliary detectors, and mass spectrometers is depicted in Fig. 3. The arrangement of branches from the Valco tee flow splitting system previously used [4] was modified slightly to provide flow to the 2D(2). A 75 μm i.d. × 2.5 m long piece of fused silica capillary (#160-2644-10, Agilent Technologies, Inc., Santa Clara, CA, USA) was connected to a Valco union via an adapting sleeve (#F-242X, IDEX Health and Science, LLC, Oak Harbor, WA, USA), with a 0.10 mm i.d. x 10 cm piece of stainless steel tubing on the distal end to attach to the switching valve (seen at the far right in Fig. 1a). The capillary length produced a flow rate of 53.6 μL/min, so that the fill time of the alternating 100 mL sample loops was 1.86 min, with a total run time (= modulation time) of 1.91 min, to exactly match the commercial 2D-LC system. Another Valco tee splitter was added after the 2D(2) UV detector (Fig. 3), to split flow between the FLD + ELSD and the LCQ Deca XP ion trap mass spectrometer operated in ESI-MS mode.
Results and discussion
There were several limitations imposed by the OLCS software that had to be worked around to enable the $^2$D(2) separation that we desired. First, the 2D-LC arrangement of instruments that was commercially available only allowed a binary UHPLC pump to act as the $^2$D separation. Therefore, the quaternary UHPLC (LC#3) was controlled separately and attached to the 4P2PVs to allow the $^2$D(2) separation, while the Agilent 1290 binary UHPLC (LC#2) was used for the $^2$D(1). Second, to accomplish the same type of shifted gradient that was allowed on the commercial 2D-LC system, each individual sub-gradient had to be manually programmed into the quaternary pump timetable. Unfortunately, the OLCS software has a limit of 100 time steps allowed in gradient timetables. Each sub-gradient was composed of five time points: (1) start initial isocratic composition, (2) end of isocratic composition and start of gradient, (3) end of gradient and start of hold composition, (4) end of hold composition and start of recycle gradient, and (5) end recycle to next isocratic composition and held until start of next sub-gradient. Therefore, the 100 allowed steps divided by 5 steps per sub-gradient permitted 20 sub-gradients per UHPLC method. Each sub-gradient was set to 1.91 min to exactly match the shifted gradient times from the commercial 2D-LC binary UHPLC. This was not required, but allowed simplified data analysis using identical parameters to those used for the $^2$D(1) in the LC x LC software (GC Image, Inc., Lincoln, NE, USA). Nevertheless, the OLCS software allowed only 20 sub-gradients that were each 1.91 min, for a total method time of 38.2 min. Therefore, two different methods with
≤ 100 steps each were required to cover the full time used for the 1D separation.

The previously reported [2, 4] first-dimension non-aqueous reversed-phase (NARP)-HPLC (LC#1) separations of TAGs were shortened to 76.4 min, to exactly match two 38.2 min 2D separation methods on the 2D(2) UHPLC. The 54 min separation of fat-soluble vitamins (FSVs) was eliminated, since natural cow’s milk is not fortified and does not contain the early-eluting vitamin D that we have analyzed in other samples. To span the length of the 1D NARP-HPLC separation, two different methods were programmed into the quaternary UHPLC (LC#3) OLCS control software, and these were joined together into a “sequence”. The first method in the sequence used the injection parameter “manual injection”, for which the start was triggered using Relay C of the Agilent 1200 HPLC (1D, LC#1) attached to the WCCCS sender board #1 (wired identically to that shown in Fig. 2). The second method in the sequence used the injection parameter “no injection”, which started the second method immediately after the end of the first method. Relay A, attached to WCCCS sender board #1 (wired to the autosampler in LC#1 as shown in Fig. 1a in an earlier report [3] and identical to Fig. 2) started the other detectors and components for the 1D (LC#1) and 2D(1) (LC#2), while relay A from LC#3 attached to WCCCS sender board #2 started the detectors for the 2D(2). We chose to use a separate relay, Relay C, to start the Agilent 1290 Infinity Flex II (LC#3) from the Agilent 1200 (LC#1) to allow more flexibility, e.g., to start the
2D(2) at a different time to analyze only a sub-section of the 1D separation, if desired. Since this report describes the hardware and software components necessary to automate the 2D(2) separation, full details of the complete set of LC gradient parameters will be described in reports of the application of this new system. At the beginning of an experiment, the 2D(2) instrument (LC#3) was started (“Run Sequence”) and all four mass spectrometers were started (via various versions of Xcalibur software), and all waited for the contact closure start signal from the 2D-LC Agilent 1200 autosampler (LC#1), via the WCCCS.

Using the reported combination of instruments, WCCCS (with sender boards, receivers, and switchable contact closure distribution manifold), TCCC, and CC-controlled ultra-high pressure valves, we were able to
accomplish the first example of the hardware and software necessary for comprehensive 2D-LC with two parallel second-dimensions \( ^2D(1) \) and \( ^2D(2) \), for \( LC1MS2 \times (LC1MS1 + LC1MS1) = LC3MS4 \). This new configuration of instruments and CC control hardware and software allows new types of automated multi-dimensional liquid chromatography for greater separation of complex samples.

Limitations

This work requires a moderate level of mechanical aptitude to accomplish construction of these components. This work requires the availability of three liquid chromatographs and four mass spectrometers.

Abbreviations

1D: first dimension; 2D: second dimension; 2D-LC: two-dimensional liquid chromatography; 4P2PV(s): 4-port, 2-position valve(s); APCI-MS: atmospheric pressure chemical ionization mass spectrometry; APPI-MS: atmospheric pressure photoionization mass spectrometry; CC(s): contact closure(s); CC control module(s); ELSD: evaporative light scattering detector; ESI-MS: electrospray ionization mass spectrometry; FLD: fluorescence detector; FSV(s): fat-soluble vitamin(s); HPLC: high performance liquid chromatography; MACM(s): microelectric actuator control module(s); NARP: non-aqueous reversed-phase; OLCS: OpenLab Chemstation; SCFA(s): short-chain fatty acid(s); TAG(s): triacylglycerol(s); TCCC: timed contact closure controlled circuit; UHPLC: ultra-high performance liquid chromatography; UIB: universal interface box; WCCCS: wireless communication contact closure controlled system.

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Authors’ contributions

This work was conceived of and carried out by the reporting author. The author read and approved the final manuscript.

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Not applicable.

Consent for publication

Not applicable.

Competing interests

The author declares that there are no competing interests.

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References

1. Byrdwell WC. Multiple parallel mass spectrometry for liquid chromatography. In: Holčapek M, Byrdwell WC, editors. Handbook of advanced chromatography/mass spectrometry techniques, Chapter 10. Champaign: Elsevier/AOCS Press; 2017. p. 365–405.
2. Byrdwell WC. Blue Jacaranda seed oil analysed using comprehensive two-dimensional liquid chromatography with quadruple parallel mass spectrometry. Chromatogr Today. 2018;591:56–64.
3. Byrdwell WC. Construction of a wireless communication contact closure system for liquid chromatography with multiple parallel mass spectrometers and other detectors. J Lab Autom. 2014;19:461–7.
4. Byrdwell WC. Comprehensive dual liquid chromatography with quadruple mass spectrometry (LC1MS2 x LC1MS2 = LC2MS4) for analysis of Parinari Curatellifolia and other seed oil triacylglycerols. Anal Chem. 2017;89:10537–46.

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