How many mass spectrometers are enough?
Adventures in multiple parallel mass spectrometry

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Analysis of lipid mixtures has always been a complicated undertaking owing to the variety of lipid classes that may be present—fatty acids (FA), triacylglycerols (TAG), phospholipids (PL), sterols (ST), and others—and the large number of molecules within each class. The variety of combinations of FA with different chain lengths and different degrees of unsaturation (i.e., molecular species) that can be present in each class is extensive, which is further complicated by the different combinations of how they are arranged on the glycerol backbone (for TAG and glycero-PL) to give different regioisomers, as well as different double bond isomers (e.g., ω-3 vs. ω-6, etc.).

- **Using two or more mass spectrometers in parallel is not impractical or expensive.**
- **Using multiple ionization methods reduces uncertainty and provides more and complementary information in less time.**
- **New methods allow analysts time to interpret data and do other high-value tasks, instead of wet chemistry.**

LC-MS IS IDEALLY SUITED TO UNRAVELING THE COMPLEXITY OF LIPIDS

Although identifying all of these possible combinations is a daunting task, technological advances have risen to the challenge and produced new generations of liquid chromatography-mass spectrometry (LC-MS) instruments and software that allow detailed lipid analysis with greater accuracy and at lower levels than ever. Advances in liquid chromatography instruments as well as column technologies provide an incredible array of options to the lipid chemist. The rise of ultra-high performance liquid chromatography (UHPLC) and the development of sub-2 μm column packings make detection of trace amounts of lipid components more feasible than ever.

Furthermore, recent generations of mass spectrometers that combine conventional mass analyzers (quadrupole, ion trap, time of flight) into new, powerful hybrid configurations (q-trap, q-TOF, etc.) allow detailed structural analyses to be accomplished that were never before possible. Even after all of these advancements, though, no single LC-MS approach is ideal for all lipids, so compromises and trade-offs must still be made.

Atmospheric pressure ionization options. At the nexus between the liquid chromatograph, with its condensed mobile phase, and the mass spectrometer, which usually requires molecules be in a gaseous state for mass analysis, lies the atmospheric pressure ionization (API) interface. Three API interfaces have risen above all others for routine use and are commercially available from most manufacturers. These are (i) atmospheric pressure chemical ionization (APCI), in which atmospheric gases or mobile phase molecules act as chemical reagents to ionize eluted molecules as they pass by a corona needle at high voltage,(ii) electrospray ionization (ESI), in which the API interface acts as an electrochemical cell maintained at high voltage to gently impart a charge (often with addition of an electrolyte to form an adduct), and (iii) atmospheric pressure photoionization (APPI), in which medium-energy photons (~10 eV) from a krypton lamp (sometimes xenon or argon) ionize a molecule, often with the help of a dopant that absorbs the light and ionizes more efficiently than the analyte and, once ionized, transfers the charge to the analyte.

Due to the different mechanisms of ionization and inherently different ionization efficiencies for different classes of molecules,

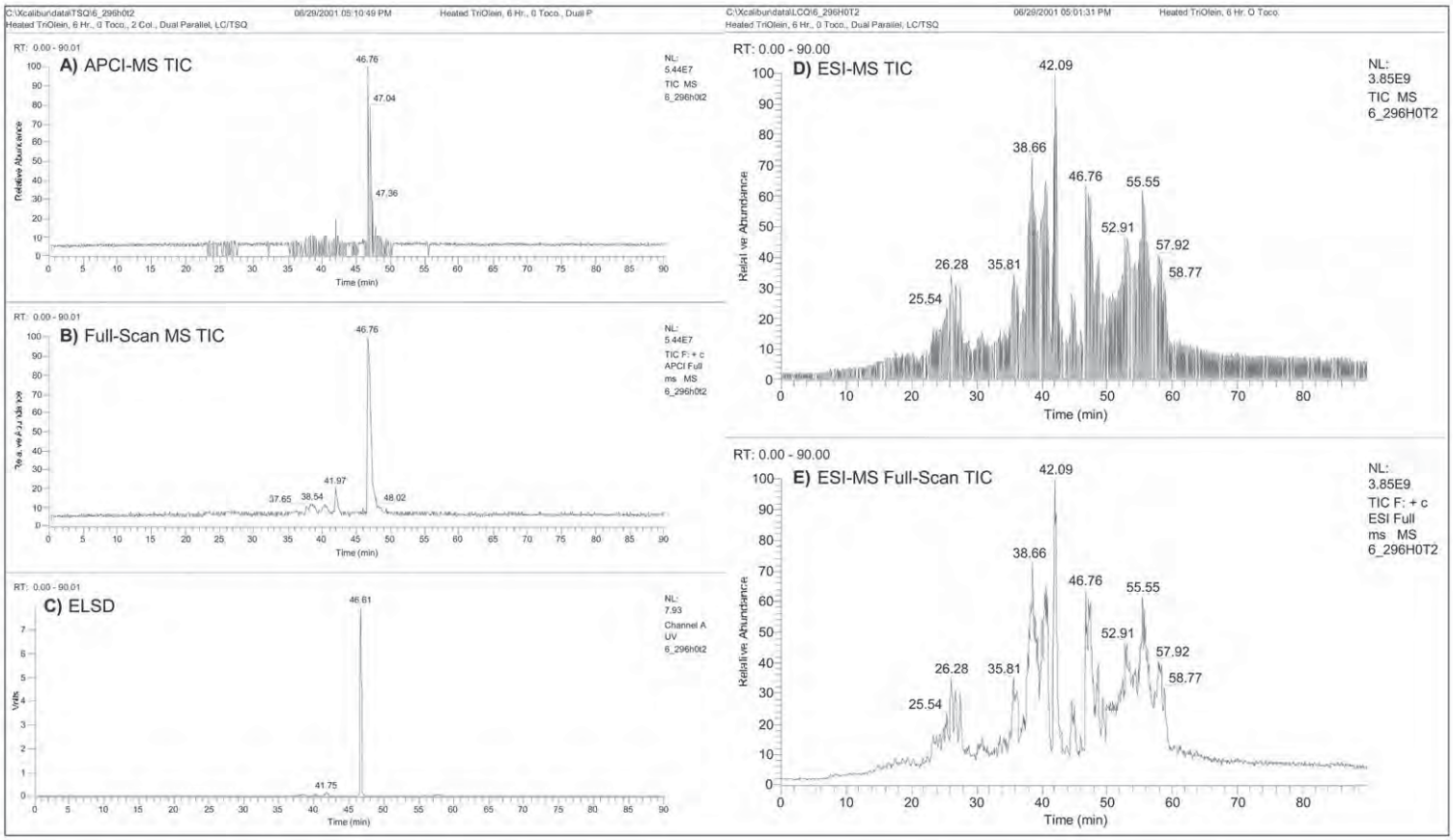

FIG. 1. *Dual parallel atmospheric pressure chemical ionization-mass spectrometry (APCI-MS) and electrospray ionization (ESI)-MS for heated triolein and its oxidation products. (A) APCI-MS with auto-MS/MS; (B) extracted ion chromatography (EIC) of APCI-MS full scans; (C) evaporative light-scattering detector (ELSD); (D) ESI-MS with auto-MS/MS; (E) EIC of full-scan ESI-MS. TIC, total ion current.*

selection of the right API technique is where the choices and compromises begin. Some molecules, such as ST, respond very well to APCI-MS but give virtually no signal by ESI-MS, whereas others, such as PL, are ideally suited to ESI-MS but yield inadequate structural information by APCI-MS. Thus, analysis of some classes of molecules may be sacrificed for the benefit of the analysis of others. This article is about overcoming the drawbacks of any one ionization type to obtain the maximum amount of information possible from MS for every lipid class present.

In our early reports on the unique sphingolipids in the human eye lens(1), we ran samples using LC-APCI-MS and then re-ran them using LC-ESI-MS, to obtain the complementary information that these API techniques produced. ESI-MS gave intact molecules and adducts that provided molecular weight information, but ESI-MS/MS of phosphocholine-containing PL often produced only the phosphocholine head group ion, with little or no information about the backbone and fatty amide chain (or fatty acyl chains for phosphatidylcholines), whereas APCI-MS gave more structural information but much lower abundances derived from the intact molecule. Thus, by obtaining both sets of information, a full picture of the structural characteristics of the molecules could be produced.

Now that API techniques have become the default methods for analysis of many classes of molecules, numerous reports fill the literature in which comparisons are made between sequential runs from complementary API techniques. A database search of "mass spectrometry" and "APPI" and "APCI" returns 100 citations, a search of "mass spectrometry" and "APPI" and "ESI" returns 102 citations, and a search of "mass spectrometry" and "APCI" and "ESI" returns 450 citations! Including APPI, APCI, and ESI together produces 64 citations, but only four seem to be applications to lipids or TAG (found by including "lipid*" or "triacylglycerol*" or "triglyceride*" as keywords). As an increasing number of researchers are finding, it is hard to overestimate the benefit of having two independent streams of MS data to allow conclusive identification of molecular species, especially those at low levels, and those for which the LC separation is less than perfect.

DUAL PARALLEL MS

After our initial report (1) in 1997, it seemed self-evident that it would be much better to obtain these data simultaneously instead

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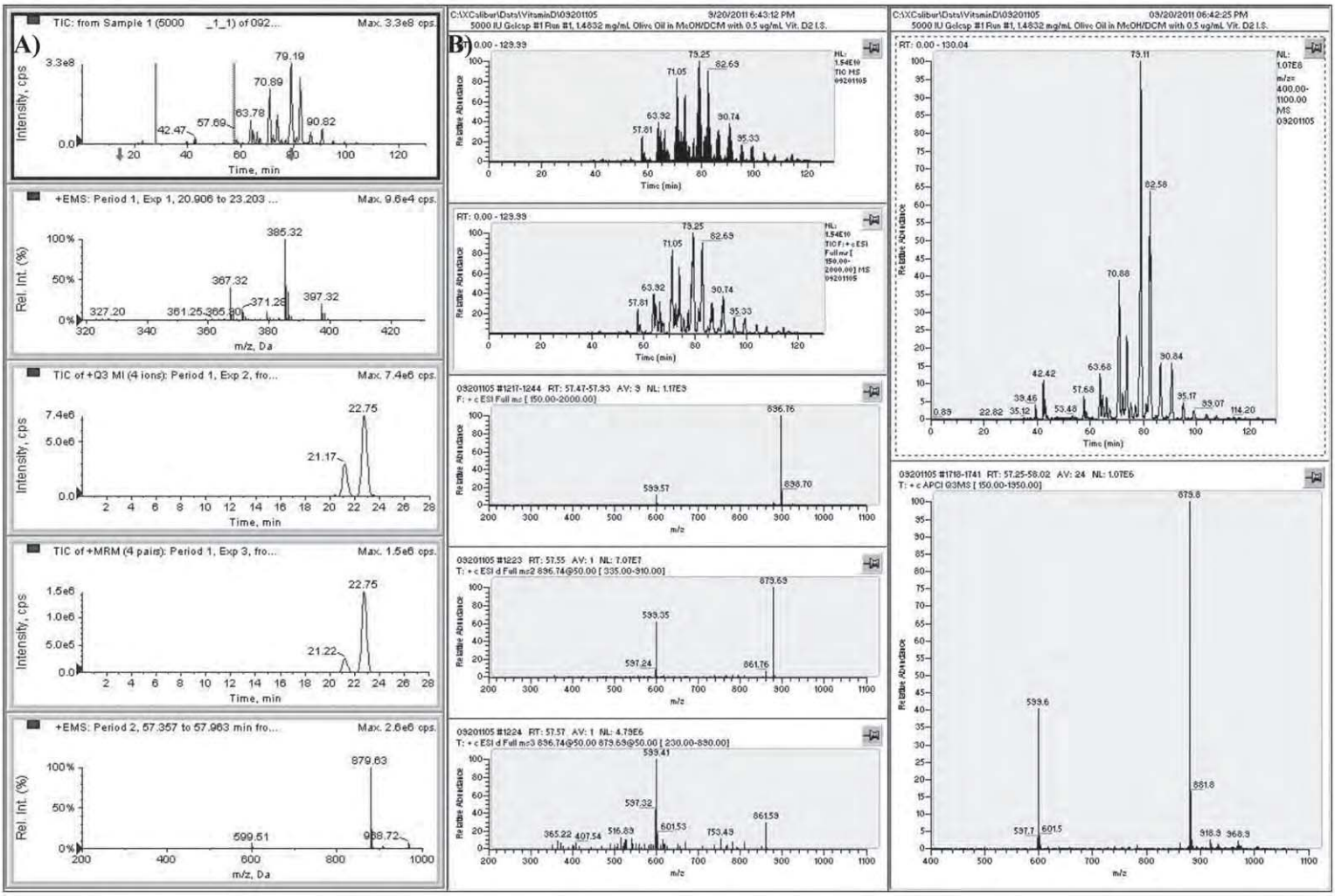

FIG. 2. *Data from triple parallel mass spectrometry analysis of olive oil-filled 5,000 international units (125 µg) vitamin D3 dietary supplements diluted to 100 mL. (A) APCI-MS with selected ion monitoring (SIM) and selected reaction monitoring (SRM) on QTrap 5500 hybrid mass spectrometer; (B) ESI-MS with auto-MS^3 on LCQ Deca XP ion trap mass spectrometer; (C) APCI-MS on TSQ 7000 tandem sector quadrupole mass spectrometer. For other abbreviations see Figure 1.*

of sequentially, so the time, solvents, and resources required for separate runs could be reduced. The next year we published our first report of the use of "dual parallel MS" for analysis of phospholipids (2). Of course, that report from 1998 now appears crude by comparison, since it pre-dated the auto-MS/MS (data-dependent acquisition, DDA) that we now take for granted in all modern MS data acquisition software. The instrument procedures for that early work had to be written manually using the instrument command language, causing the cycle times to be much slower than would be acceptable on a modern instrument. Although crude, those data effectively demonstrated that the dual parallel MS approach was beneficial and that such experiments could be accomplished using simple off-the-shelf components, without the need for extensive prototyping (a figure showing the first phospholipids analysis using atmospheric pressure chemical ionization-mass spectrometry and electrospray ionization simultaneously is available in the supplement to the digital edition, which can be accessed by logging in to read the May 2013 issue at www.aocs.org/login). Splitting of the effluent stream was accomplished using simple tees, with the flow to each instrument mainly dictated by the length and internal diameter of the capillary tubing used for each instrument. To be thorough, data from an ultraviolet (UV) detector and an evaporative light scattering detector (ELSD) were also acquired.

Since that initial report, we have used multiple ionization techniques in parallel whenever possible. Applications of dual parallel MS up to 2005 were presented in the AOCS Press book *Modern Method for Lipid Analysis* (3). One of the best and most cited examples was the use of dual parallel MS for analysis of TAG and TAG oxidation products (TAGOX). Those data, pictured in Figure 1 (page 313), dramatically demonstrate the benefit of ESI-MS of ammonium adducts for TAGOX analysis. TAGOX responded much more sensitively to ESI-MS than APCI-MS, while the ELSD showed that APCI-MS discriminated between classes less than ESI-MS. At the same time, APCI-MS provided valuable fragments

Unique parallel mass spectrometry methods for vitamin D and triacylglycerol analysis

Potential links between vitamin D deficiency and numerous health problems beyond bone health have been identified recently. Awareness of the importance of maintaining adequate vitamin D levels is increasing among consumers and scientists alike.

Existing methods for vitamin D analysis are very labor intensive and time consuming, and there is a great deal of variability in results in the literature. Most methods are similar to those that have been used for decades. They involve saponification—to break down triacylglycerols (TAG), a potential interferent—followed by liquid/liquid extraction, semipreparative chromatography, fraction collection, evaporation and reconstitution, and finally, analytical chromatography. We were quite surprised when we found that, even after all of that sample preparation, including two chromatography steps, some samples, such as processed cheese and orange juice, still showed interfering species that co-eluted with vitamin D in the UV (ultraviolet) chromatograms. In some cases, the co-elution produced nice-looking symmetric peaks that did not belie the underlying troublemaker; but, we were not fooled.

PARALLEL MS FOR VITAMIN D

Based on prior experience, it was only natural to apply dual parallel mass spectrometry (MS) to the analysis of vitamin D in foods. One instrument was dedicated to acquisition of targeted ions for quantification of vitamin D by selected ion monitoring (SIM) atmospheric pressure chemical ionization mass spectrometry (APCI-MS), while a second instrument was used as a "watchdog" for qualitative analysis using full-scan APCI-MS followed by auto-MS/MS. Not only could we see that some samples had peaks that overlapped in the UV chromatograms, we could also identify the masses associated with the interferent(s). For those samples, the UV data could be ignored, and quantification of vitamin D could be accomplished by SIM APCI-MS. For other samples, we had MS data to prove that no interferent was present, so the more sensitive results by UV detection could be trusted. Nowadays, we never trust peaks in UV chromatograms unless there are full-scan MS data to conclusively prove the absence of interfering species.

TRIPLE PARALLEL MS AND MORE

In 2011 we reported the results of a new "dilute-and-shoot" triple parallel MS experiment in which we were able to no longer treat the TAG as interfering species that needed to be broken down and eliminated, but instead were able to perform a holistic analysis of the samples in which both the vitamin D and the composition of the TAG in the bulk oil (rice bran oil in that case) could be determined (*Anal. Bioanal. Chem. 401*:3317–3334). Typical data from three mass spectrometers in a dilute-and-shoot triple parallel mass spectrometer experiment applied to a 5,000 IU vitamin D3 dietary supplement in olive oil are shown in Figure 2 (see main text). Virtually all sample preparation was eliminated, and a single chromatographic system was used for separation of vitamin D2 and D3, as well as pre-vitamin D2 and pre-vitamin D3, if present.

We have now added the capability to perform atmospheric pressure photoionization (APPI)-MS on either of two instruments, in addition to high- and low-sensitivity APCI-MS and electrospray ionization-MS, for quadruple parallel MS, as well as the corona charged aerosol detector, evaporative light-scattering detector, and UV data, for seven detectors overall. We are working to expand the applicability of the dilute-and-shoot approach to other samples.

for structure elucidation, although unusual fragmentation pathways for TAGOX required complementary ESI-MS/MS data to avoid potential misinterpretation.

Such approaches can be implemented by practically any determined analytical chemist, especially after upgrading an instrument in a laboratory. An older instrument that is being replaced can be pressed into service as an auxiliary detector, while at the same time taking advantage of the sensitivity and scan functions available on a newer instrument. Although it is not practical to buy two new mass spectrometers to implement a dual parallel MS approach, it can easily be accomplished during the natural evolution and upgrade of a laboratory over time. Furthermore, used instruments are available at such reasonable cost that the ability to incorporate an additional mass spectrometer is no longer a serious obstacle.

TAKING IT TO THE NEXT LEVEL(S)

In May 2011 in the AOCS Press book *Extreme Chromatography* (4) and in an article published later that year (5), the bar was raised to the next higher level as the first "triple parallel MS" experiment was reported. Typical data for an olive oil-filled dietary supplement containing 5,000 international units (IU) (=125 μg) of vitamin D3 are shown in Figure 2. Previous examples showed the results of the analysis of supplements containing rice bran oil (5) and sunflower oil (4). Vitamin D3 present in dietary supplements at low levels (limit of detection, ~25–90 ng/mL) (5) was analyzed using APCI-MS and UV detection, but the composition of the oil in which the vitamin was dissolved was also determined. Unfortunately, the TAG in the oil overwhelmed the mass analyzer of the newest and most sensitive instrument and led to incorrect isotope ratios and incorrect masses due to improper centroiding (converting a peak profile to a single line at the center of mass). However, an older, less sensitive instrument was ideal for TAG analysis and gave reliable results similar to those obtained in preceding years. Thus, we used high-sensitivity APCI-MS for vitamin D and lower-sensitivity APCI-MS for the bulk oil to accomplish a holistic

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analysis of the supplements. We obtained ESI-MS results simultaneously in parallel that were similar to those obtained in the past, which again provided a valuable complement to the APCI-MS data. UV (single channel and scans), ELSD, and a corona charged aerosol detector (CAD) were also used, for six detectors, overall. Was that enough? Not yet.

We are currently preparing a report on "quadruple parallel MS," using a different combination of instruments. We have added the capability to perform APPI-MS, in addition to high- and low-sensitivity APCI-MS and ESI-MS, having exchanged the instrument on which high-sensitivity APCI-MS was conducted, and adding a hybrid instrument with a higher mass range than the one used previously. We also used an upgraded corona CAD, as well as the ELSD and UV detector, for seven detectors overall. A contact closure distribution panel built from speaker wire and low-cost switches allows different LC systems and MS instruments and other detectors to be reconfigured into a variety of experiments, with the flips of a few switches (photo available in the supplement to the digital edition, which can be accessed by logging in to read the May 2013 issue at www.aocs.org/login).

TIPS AND TRICKS OF THE TRADE

These experiments have allowed us to draw some conclusions about the potential and reality of using multiple complementary mass spectrometers in parallel and to make suggestions regarding their use:

1. Multiple parallel MS can be accomplished by any determined analyst with more than one instrument at his/her disposal using simple off-the-shelf components.
2. Using instruments in parallel saves time, labor, and resources compared to performing sequential runs using different ionization methods and eliminates uncertainty due to run-to-run variability between chromatographic runs.
3. Older instruments can provide a valuable contribution to multiple parallel MS experiments as auxiliary detectors and should be kept in service as long as possible; also, used instruments are very affordable.
4. APCI, APPI, and ESI provide different and complementary structural information on various classes of lipid molecules.
5. Conventional two-dimensional detectors (UV, ELSD, corona CAD) are easily incorporated into multiple parallel MS experiments, and UV is more sensitive than APCI-MS for some classes.
6. Conventional analytical-scale HPLC is more suited to parallel MS than UHPLC because it provides enough flow to split among multiple instruments, peak widths are more compatible with scanning speeds of older instruments, and it allows more DDA experiments across a peak.
7. Some ionization types discriminate between classes more than others (especially ESI), while others show noticeable differences between molecular species within classes.
8. Even within the same type of API source, sources from various manufacturers behave differently and give different signal-to-noise ratios, and some brands discriminate between molecular species more than others.
9. It is helpful to synchronize the system clocks on multiple instruments to minimize minor differences in times shown in figures.
10. It is beneficial to show the time in data headers in figures to show that data were obtained in parallel.

We can finally ask: "Are four mass spectrometers in parallel enough?" The experiments described above have all used one liquid chromatograph with two to four mass spectrometers (LC1/MS2 to LC1/MS4). However, we have also used two liquid chromatographs, with one mass spectrometer attached to each chromatograph, in an LC2/MS2 column-switching experiment in which both polar and nonpolar lipids were simultaneously analyzed on two different LC-MS systems from one injection (6). It is easy to imagine the possibility that APCI, APPI, and ESI could each be used in parallel for detection of the effluent from each of the two LC systems, in an LC2/MS6 experiment (plus UV, ELSD, and CAD, of course). For me, that would probably be "enough"! Or do we need high- and low- sensitivity APCI-MS on one or both? LC2/MS8?

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