Failure to apply standard limit-of-detection or limit-of-quantitation criteria to specialized pro-resolving mediator analysis incorrectly characterizes their presence in biological samples.

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Matters Arising: Nature Communications (under consideration) on Gomez EA, Colas RA, Souza PR, Hands R, Lewis MJ, Bessant C, Pitzalis C and Dalli J. Blood pro-resolving mediators are linked with synovial pathology and are predictive of DMARD responsiveness in rheumatoid arthritis. *Nat Commun*. 2020;11:5420.

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Summary.

Specialized pro-resolving mediators (SPM) derived from oxygenation of long chain polyunsaturated fatty acids (PUFA) were originally described by Serhan and colleagues and have been proposed as mediators of inflammation resolution. Families of SPM described in the literature include lipoxins, resolvins, maresins, protectins and their peptide conjugates. In this article Gomez and co-authors report that levels of plasma SPM from patients with early rheumatoid arthritis predict response to biologic therapy after 6 months. SPM were measured in this study using liquid chromatography tandem mass spectrometry (LC-MS/MS). On reviewing the methods, supplementary analytical data and the online peer review file, we note several concerns, regarding both analytical methods and experimental conclusions. Specifically, quantifications of multiple SPM are based on weak or absent peaks in ion chromatograms, and mass spectra extracted from the recordings do not concord with authentic standards of SPM molecules presented. Indeed, applying their methodology, it is possible to infer the presence of lipids in clean methanol and buffer blanks. The LC-MS/MS analyses described, which are widely applied to the analysis of SPM, fail to meet the standards of accepted practices in the small molecule and lipidomics field or those endorsed by professional bodies for analytical chemistry. Application of this flawed methodology to SPM analysis brings into question the very occurrence of many of these lipids in biological samples, their proposed impact on inflammatory processes, and biomarker claims.

Main Text.

In this study, Gomez et al[1] override a signal-to-noise ratio (S/N) criterion (*"it does not form part of our core identification criteria")*, and cite the following as the basis for identification and quantification:

"(*1) presence of a peak with a minimum area of 2000 counts, (2) matching retention time to synthetic or authentic standards with maximum drift between the expected retention time and the observed retention time of 0.05 s, (3) ≥4 data points, and (4) matching of at least 6 diagnostic ions to that of reference standard, with a minimum of one backbone fragment being identified in representative samples"*.

Thus, they do not include the established approach in analytical guidelines from multiple agencies for calculating S/N ratio and using this to set the limits of detection (LOD) and limit of quantitation (LOQ). This is the approved methodology set by the International Conference on Harmonisation (ICH), the European Pharmacopoeia (Ph. Eur), the International Organisation for Standardisation (ISO), the European Medicines Agency (EMA), the Food and Drug Administration (FDA), the United States Pharmacopeia (USP), the International Union of Pure and Applied Chemistry (IUPAC), and the World Health Organisation (WHO)[2-11]. While validated assays have recommended S/N of around 10 for LOQ, and 3:1 for LOD[2], the practice of many academic research labs has been to use around 5:1 for LOQ. Consistent with this, the FDA requires that the analyte response at the LOQ should be ≥ 5 times the analyte response of the zero calibrator[12]. Noting this, our concerns led us to assess the validity of the method experimentally, focusing particularly on points 1 and 4.

Regarding Gomez criterion "*presence of a peak with a minimum area of 2000 counts":*
The Sciex mass spectrometer used in the authors' analyses computes peak areas as *counts per second* (cps) in ion chromatograms. However, these instruments are exquisitely sensitive, and we found they can generate >2000 cps from blank recordings, as illustrated from an experiment using a similar model Sciex instrument in one of our laboratories. Here, RvD2 was monitored using three injections of HPLC-grade methanol and all integrations at the relevant retention time returned values >2000 cps (Figure 1B). Similar results were found for 4 other oxylipins (Supplementary Figures 1, 2A). Thus, integration of methanol chromatograms at the expected retention time (where no discernible peak exists) generated cps areas that exceed 2000 for several oxylipins. We conclude that this is not a suitable approach for deciding whether a lipid is present in a sample because (i) this value can be exceeded in blank injections in the absence of a visible peak, (ii) it does not take into account the variability of noise. Since background noise levels in biological samples will be higher than for neat standards (matrix effect) and varies between different multiple reaction monitoring (MRM) channels, this issue will be magnified significantly when analysing tissue extracts.

Prompted by our findings, we reviewed the chromatographic peaks published in Gomez et al, Supplementary Data[1] and selected six which illustrate highly unsatisfactory S/N ratios: PDx, PCTR1, RvD6, Mar1, RvT2, 5S,15S-diHETE (Figure 1C). By any conventional standards, these analyses are representative of false positives. In these and others (e.g., RvD5, 17R-RvD1, 17R-RvD3, PCTR2, 22-OH-MaR1, 14-oxo-MaR1, 7S,14S-diHDHA, MCTR2, RvT4, RvD5(n-3 DPA), PD1(n-3 DPA), MaR1(n-3 DPA), LXA4, 15R-LXB4, and TXB2), the S/N ratio in the biological

samples by our estimation is <5, providing no acceptable basis for quantitation. In the shown examples, there is no discernible peak at all in the sample, meaning that this represents integration of simple baseline noise. To evaluate peak quality further, we inspected the peer reviewed articles cited by Gomez et al as examples of this long-published SPM analytical method, which comes from the Serhan group[13-20]. Five show figures of chromatographic peaks which do not appear to be primary data, thus their underlying raw data peaks could not be evaluated[13, 15, 17, 19, 20] (for examples see: Fig 2A[20], Supplemental Fig 1A[13]). We refer the reader to the peer review file where this issue was also noted (page 13 of supplementary file[1]).

Regarding the Gomez criterion "*matching of at least 6 diagnostic ions to that of reference standard, with a minimum of one backbone fragment being identified in representative samples*".
In standard practice, for a positive match the MS/MS spectra of standard and sample should be visually similar when recorded on the same instrument using the same parameters; the dominant product ions should be present in both with a generally similar pattern of relative abundance. Considering the data in Gomez et al and taking the very first analysis in the Supplementary Figures as an example, the spectra of RvD1 standard and sample are weak and noisy; there are different relative abundances of presumed "diagnostic" product ions and the large prominent RvD1 ion that should be seen at *m/z* 215 is not distinguishable from background noise (Figure 2 A). A clean standard MS/MS is shown for comparison, obtained in one of our laboratories on a 6500 QTrap instrument (Figure 2 B). Due to space limitations, we are unable to discuss similarly all sample spectra in this article. However, many product ion spectra in the Supplement (a) do not match their standards, (b) are of poor quality and noisy and (c) their use for identification purposes is incorrect and misleading. Overall, we assert that these MS/MS data do not support the presence of SPMs in the biological samples. From experience, we stress that when lipids are present *in very low amounts* in a biological sample, MS/MS spectra are often inconclusive and do not compare well with standards. If poor quality MS/MS spectra are used in this manner, the approach becomes fundamentally flawed.

We next looked at MS/MS data in papers cited by Gomez in support of the method[13-20]. Three papers report RvD1 and RvD3 in serum or synovial samples from mice or humans [13, 15, 20]. There is a lack of similarity between spectra for either lipid across these studies, apart from the selection of low abundance ions, many indistinguishable from background. In one article, there are 2 MS/MS spectra for RvD3, in mouse paws or human serum (Figures 1, 3 respectively[13]) These are different from each other and from the MS/MS spectra in the other two articles. Importantly, these bear no resemblance to the MoNA reference spectrum (https://mona.fiehnlab.ucdavis.edu/spectra/display/IA000269), which has ions at *m/z* 69, 95, 115, 137 and a large prominent ion at 147.

The newer generation MS platforms including the Sciex QTrap instruments are all highly sensitive, and potentially capable of detecting "ions" from the laboratory, solvent, or simple electronic noise that might correspond to potentially "diagnostic" ions in the analyte in question. This is illustrated by an experiment conducted in one of our laboratories in which phosphate buffered saline was processed using solid phase extraction (see Methods) and analysed using LC-MS/MS while acquiring enhanced product ion (EPI) MS/MS spectra throughout the analysis. Analysis of hypothetical Mar1 shows an absence of a chromatographic peak when monitoring *m/z* 359-250, as expected (Figure 2 C). However, at

the retention time for Mar1 (and throughout the entire run), the instrument could isolate a signal from buffer at *m/z* 359 which could be fragmented into a series of product ions (Figure 2 D). Zooming into this, following conversion of the spectrum from profile to centroid by the instrument software *Analyst*, many ions are seen including several (*m/z* 113, 141, 221, 297, 315, 323, 341) that match well the "diagnostic" ions shown in Supplementary Figure 1P, Gomez et al (Figure 2 D,E). Furthermore, low intensity ions at *m/z* 113 or 141 originating from fragmentation of *m/z* 359 are detected across the entire chromatographic run (Figure 2F). The first described literature reference spectrum for Mar1 is reproduced for reference (Figure 2G)[21]. The unique even mass product ion of *m/z* 250 which was deemed "diagnostic" for that DHA metabolite[21] is absent in the "Sample" and "Standard" product ion mass spectra presented by Gomez et al. in Supplementary Figure 1P[1]. Similarly, analysis of extracted PBS for several putative resolvin precursor-to-product ion transitions could isolate and fragment signals at the expected precursor masses, which could in turn generate variable signals of purported "diagnostic" product ions (Supplementary Figures 2 B,C, 3). Here, we used PBS, while complex lipid extracts from tissue would have a far higher level of background noise ions to contribute to MS/MS. This experiment affirms that high sensitivity mass spectrometers can be set up to generate what appear to be poor quality MS/MS "spectra" from blank samples, which can be analysed to incorrectly infer the presence of SPM [1, 13-20].

Conclusions

In summary, we show that that chromatograms and "ghost" spectra from methanol or extracted buffer blanks can generate integrated areas in excess of 2000 cps, as well as poor quality MS/MS data with evidence suggesting precursor and product ions that are seen in SPM datasets from Gomez et al[1]. Our view is that the method used by Gomez[1] and the cited articles[13-20] is flawed, and can artefactually detect lipids where none exist. More generally, since this is the method most commonly applied to SPM analysis in the literature, the evidence for the presence of SPM in biological matrices and an inferred role in the resolution of inflammation needs to be reassessed.

Methods

Newly opened SPE cartridges (Waters, Sep-Pak C18, 6 mL capacity, 500 mg) were conditioned with 5 mL of methanol (Fischer, HPLC grade) followed by 10 mL of Ultrapure water (Cayman). 5 mL of phosphate buffered saline was then loaded and columns washed with 10 mL of Ultrapure water. 3 mL ethyl acetate (Sigma-Aldrich, LC-MS grade) was used to elute oxylipins. This was evaporated under vacuum and samples re-dissolved in methanol, before being analysed using LC-MS/MS as described. LC-MS/MS was performed on a Nexera liquid chromatography system (Nexera X2, Shimadzu) coupled to a 6500 QTrap mass spectrometer (AB Sciex). Liquid chromatography was performed at 45 °C using a Zorbax Eclipse Plus C18 (Agilent Technologies) reversed phase column (150 × 2.1 mm, 1.8 µm) at a flow rate of 0.5 mL/min over 22.5 min. Mobile phase A was (95 % HPLC water/5 % mobile phase B; v/v and 0.1 % acetic acid) and mobile phase B was acetonitrile/methanol (800 ml + 150 ml; and 0.1 % acetic acid). The following linear gradient for mobile phase B was applied: 30 % for 1 min, 30 - 35 % from 1 to 4 min, 35 – 67.5 % from 4 to 12.5 min, 67.5 – 100 % from 12.5 to 17.5 min and held at 100 % for 3.5 min, followed by 1.5 min at initial condition for column re-equilibration. Injection volume was 5 µL. Lipids were analyzed in monitoring (MRM) mode with scheduling (55s) for the baseline integration experiment. Ionization was performed using electrospray ionization in the negative ion mode with the following MS parameters: temperature 475 °C, N_2 gas, GS1 60 psi, GS2 60 psi, curtain gas 35 psi, ESI voltage -4.5 kV. Cycle time was 0.4 s. For MS/MS analysis, enhanced product ion mode was used with dynamic fill time. Data were integrated using Analyst software. Data showing integrated windows are shown as screenshots, while MS/MS analysis was copied into PowerPoint for minimal processing (linewidths, font sizes only) with no alterations to chromatographic or MS/MS data. MS/MS is presented as profile or centroid as described in Figure Legends. Oxylipin standards were from Cayman Chemical.

Figure Legends

Figure 1. Analysis of methanol blanks shows integrated peak areas >2000 cps for RvD2, while flawed S/N analysis shows false positives for several SPM in Gomez et al. Panel A. Example chromatogram from 1.8 ng RvD2 standard analysed using LC-MS/MS as described in Methods. Panel B. Three separate analyses of a methanol injection, in the region where RvD2 elutes showing the areas where the signal was integrated. Panel C. Chromatograms from Supplementary information. The chromatograms are representative of many from Supplementary information showing the authentic standard on top with analysis of the biological sample immediately below. In BLUE are the peaks areas computed by Gomez and the green strips are the regions the authors used to calculate S/N ratios of 4, 4, 5, 7, 6 and 5, reading clockwise from the top left panel. In Red, added to the originals, are labels of Standard, Biological Sample, a box around the original S/N, and estimations of S/N <2, considering the SPM signal and the entire available baseline.

Figure 2. MS/MS of RvD1 do not match between standard and sample, and an extracted buffer blank shows absence of peak, but several detectable "diagnostic" ions for Mar1, with some being detected throughout the entire run. Panel A. Screenshot of Supplementary Figure 1A showing MS/MS of standard and sample, from Gomez et al. Panel B. MS/MS of an RvD1 standard generated in one of our laboratories. Panel C. Chromatogram, monitoring for Mar1 at

m/z 359-250. Panel D. MS/MS at 10-10.2 min, where the Mar1 standard elutes, showing isolation and fragmentation of ion at *m/z* 359. Panel E. Zoomed in regions of centroid spectrum showing background ions contain several "diagnostic" ions for Mar1, as labelled by red arrows. Panel F. Ions at m/z 113 or 141 that are detected following fragmentation of m/z 359 are detected throughout the chromatographic run. Panel G. Figure showing first report of Mar1 MS/MS spectrum from[21]. Panel G. Reproduced from Serhan et al., 2009[21], the first report of maresin-1 (Mar1) as a novel metabolite of DHA.

Supplementary Figure 1. Further examples where baseline noise integration generates signals higher than 2000 cps. Panel A. Example chromatogram from LTB3 standard analysed using LC-MS/MS as described in Methods. Three separate analyses of a methanol injection, in the region where LTB3 elutes showing the areas where the signal was integrated. Panel B. Example chromatogram from 8-HETE standard analysed using LC-MS/MS as described in Methods. Three separate analyses of a methanol injection, in the region where 8-HETE elutes showing the areas where the signal was integrated. Panel C. Example chromatogram from 15-HETE standard analysed using LC-MS/MS as described in Methods. Two separate analyses of a methanol injection, in the region where 15-HETE elutes showing the areas where the signal was integrated.

Supplementary Figure 2. Examples of baseline noise integration and the presence of false "diagnostic" ions in MS/MS from extracted buffer blanks. Panel A. Example chromatogram from 5-HETE standard analysed using LC-MS/MS as described in Methods. Analysis of a methanol injection, in the region where 5-HETE elutes showing the areas where the signal was integrated. Panel B. Chromatogram, monitoring for RvD1 at *m/z* 375-215 in standard and blank. Panel C. MS/MS at 7.2-7.4 min, where the RvD1 standard elutes, showing isolation and fragmentation of ion at *m/z* 375. Zoomed in regions of centroid spectrum showing background ions incorrectly identified as "diagnostic" ions for RvD1, as labelled by red arrows.

Supplementary Figure 3. The presence of putative "diagnostic" ions in MS/MS from extracted buffer blanks Panel A. Chromatogram, monitoring for RvD5 at *m/z* 359-199 in standard and blank. Panel C. MS/MS at 9.9-10.1 min, where the RvD5 standard elutes, showing isolation and fragmentation of ion at *m/z* 359. Zoomed in regions of centroid spectrum affirming that background ions can be incorrectly identified as "diagnostic" ions for RvD5 (red arrows).

Author conflicts

BAF acknowledges an interest in Creegh Pharmaceuticals, Inc.
ARB is a consultant for Lonza Pharma and Biotech.
GAF is Senior Advisor to Calico Laboratories.

References

1. Gomez EA, Colas RA, Souza PR, Hands R, Lewis MJ, Bessant C, Pitzalis C and Dalli J. Blood pro-resolving mediators are linked with synovial pathology and are predictive of DMARD responsiveness in rheumatoid arthritis. *Nat Commun*. 2020;11:5420.
2. https://www.ema.europa.eu/en/ich-q2-r1-validation-analytical-procedures-text-methodology.

3.https://www.who.int/medicines/areas/quality_safety/quality_assurance/28092018Guideline_Validation_AnalyticalMethodValidation-Appendix4_QAS16-671.pdf.

4. http://www.pharmacopeia.cn/v29240/usp29nf24s0_c1225.html.
5. Heyden YVB, R. *The limit of detection*. https://www.researchgate.net/profile/Stefano_Polesello/post/How_to_calculate_LOD_and_LOQ_of_analyte_by_hplc/attachment/59d6356379197b8077992f7e/AS:384058174525440@1468578012222/download/The+Limit+of+Detection-LCGC.doc: LCGC Europe; 2009.
6. Wenzl T, Haedrich, J, Schaechtele, A, Robouch, P, Stroka, J. Guidance Document on the Estimation of LOD and LOQ for Measurements in the Field of Contaminants in Feed and Food. *EUR 28099; Publications Office of the European Union: Luxembourg,*. 2016;https://publications.jrc.ec.europa.eu/repository/bitstream/JRC102946/eur%202809 9%20en_lod%20loq%20guidance%20document.pdf.
7. Araujo P. Key aspects of analytical method validation and linearity evaluation. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2009;877:2224-34.
8. Desimoni E, Brunetti, B. Signal to noise ratio; Limit of detection; Standard error of the regression; Chromatographic/Voltammetric/Spectroscopic signals. *PHARMACEUTICA ANALYTICA ACTA* 2015;6.
9. Commission E. 2002/657/EC: Commission Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (Text with EEA relevance) (notified under document number C(2002) 3044) *https://opeuropaeu/en/publication-detail/-/publication/ed928116-a955-4a84-b10a-cf7a82bad858/language-en*. 2002.
10. Research CfDEa and Research CfBEa. https://www.fda.gov/regulatory-information/search-fda-guidance-documents/analytical-procedures-and-methods-validation-drugs-and-biologics. *https://wwwfdagov/regulatory-information/search-fda-guidance-documents/analytical-procedures-and-methods-validation-drugs-and-biologics*. 2015.
11. Olivieri A FN, Ferre J, Boque R, Kalivas JH, Mark H. UNCERTAINTY ESTIMATION AND FIGURES OF MERIT FOR MULTIVARIATE CALIBRATION, IUPAC Technical Report. *Pure Appl Chem*. 78:633-661.
12. FDA U. Bioanalytical Method Validation Guidance for Industry. *FDA-2013-D-1020*.
13. Arnardottir HH, Dalli J, Norling LV, Colas RA, Perretti M and Serhan CN. Resolvin D3 Is Dysregulated in Arthritis and Reduces Arthritic Inflammation. *Journal of immunology*. 2016;197:2362-8.
14. Chiang N, Dalli J, Colas RA and Serhan CN. Identification of resolvin D2 receptor mediating resolution of infections and organ protection. *The Journal of experimental medicine*. 2015;212:1203-17.
15. Colas RA, Shinohara M, Dalli J, Chiang N and Serhan CN. Identification and signature profiles for pro-resolving and inflammatory lipid mediators in human tissue. *Am J Physiol Cell Physiol*. 2014;307:C39-54.
16. Dalli J, Chiang N and Serhan CN. Elucidation of novel 13-series resolvins that increase with atorvastatin and clear infections. *Nat Med*. 2015;21:1071-5.

17. Dalli J, Ramon S, Norris PC, Colas RA and Serhan CN. Novel proresolving and tissue-regenerative resolvin and protectin sulfido-conjugated pathways. *FASEB J*. 2015;29:2120-36.
18. Fredman G, Ozcan L, Spolitu S, Hellmann J, Spite M, Backs J and Tabas I. Resolvin D1 limits 5-lipoxygenase nuclear localization and leukotriene B4 synthesis by inhibiting a calcium-activated kinase pathway. *Proceedings of the National Academy of Sciences of the United States of America*. 2014;111:14530-5.
19. Freire MO, Dalli J, Serhan CN and Van Dyke TE. Neutrophil Resolvin E1 Receptor Expression and Function in Type 2 Diabetes. *Journal of immunology*. 2017;198:718-728.
20. Norling LV, Headland SE, Dalli J, Arnardottir HH, Haworth O, Jones HR, Irimia D, Serhan CN and Perretti M. Proresolving and cartilage-protective actions of resolvin D1 in inflammatory arthritis. *JCI Insight*. 2016;1:e85922.
21. Serhan CN, Yang R, Martinod K, Kasuga K, Pillai PS, Porter TF, Oh SF and Spite M. Maresins: novel macrophage mediators with potent antiinflammatory and proresolving actions. *The Journal of experimental medicine*. 2009;206:15-23.