

Technical note

**Comparing the quantitation of specialized pro-resolving mediators in plasma and serum using ELISA and LC-MS/MS.**

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## Abstract

Quantitation of oxylipins is generally performed using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). Researchers can also use commercial enzyme-linked immunosorbent assays (ELISAs), due to convenience or where access to instrumentation is limited. However, oxylipins are small molecules with many isomers and metabolites that are difficult to fully discriminate using ELISAs, with many potential sources of cross-reactivity. In this paper, the use of ELISAs for oxylipin analysis is compared with LC-MS/MS, with two specialized pro-resolving mediators (SPM) as examples. By reviewing the literature, we show that ELISAs report significantly higher levels of resolvin D1 (RvD1) and resolvin D2 (RvD2) than LC-MS/MS. Also, we show experimentally that in plasma and serum samples where these lipids were not detected using LC-MS/MS, a positive result could be obtained using ELISA, and that these signals increase with improper sample processing. In conclusion, while ELISA can be a valuable screening tool for the presence of oxylipins, positive signals need validation using LC-MS/MS.

## Introduction

For routine, sensitive quantitation of small molecules including lipids, liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) is the gold standard approach. When analyzing oxylipins, biologically active oxidation metabolites of polyunsaturated fatty acids, the newer generation MS platforms that incorporate rapid scanning and retention-time scheduling allow the quantitation of over 200 lipids in around 20 minutes, down to a sensitivity of low picogram (pg) levels on column<sup>1,2</sup>. This has revolutionized our ability to identify and quantify oxylipins; however, it requires a high investment in cost, time, and expertise. Since the 1970s, immunoreactivity-based methods such as enzyme-linked immunosorbent assay (ELISA) or radioimmunoassay (RIA) have been developed, first for well-known abundant oxylipins such as prostaglandin (PG) E<sub>2</sub> (PGE<sub>2</sub>) or thromboxane B<sub>2</sub> (TXB<sub>2</sub>), and later for less abundant oxylipins including specialized pro-resolving mediators (SPM) such as resolvins.

ELISA methods for PG analysis were developed decades ago<sup>3,4</sup>, allowing quantitation without the need for specialists or expensive equipment. During the 1980s, the quantitative accuracy of immunoassays was questioned. For example, comparison of two RIAs with GC-MS for 6-keto-PGF<sub>1α</sub> showed approximately 10-fold higher levels by RIA<sup>5</sup>. Another study reporting isoprostane analysis by ELISA led to the conclusion that 8-epi-PGF<sub>2α</sub> was a major vascular metabolite of prostacyclin<sup>6</sup>, a fact that was later disproved<sup>7</sup>. In contrast, RIA was successfully used to measure urine levels of 6-keto-PGF<sub>1α</sub> and TXB<sub>2</sub>, although rigorous purification was required prior to conducting the RIA to minimize cross-reactivity<sup>8,9</sup>. Recently, two studies showed how ELISAs overestimated PGE<sub>2</sub> levels in macrophage cultures, although the ELISA signal was sensitive to cyclooxygenase blockade, indicating that it reported on the PG pathway overall, therefore allowing the assay to be used to identify enzyme inhibitors<sup>10,11</sup>. Thus, ELISA can provide a useful screen for oxylipin pathways or identify differences that can then be verified using LC-MS/MS. Although ELISA vendors provide information on the cross reactivity of similar oxylipins, a comprehensive panel cannot be tested in each ELISA. Therefore, cross-reactivity of unknown species in complex samples can still take place, and it is advised that validation should be performed to confirm the presence of the analytes of interest. Thus, as stated years ago, ELISA results should be considered *semiquantitative indices of PG-like immunoactivity, unless stringently proven to be measuring the actual metabolite of interest*<sup>7</sup>. However, unfortunately, most published studies that claim detectable amounts of oxylipins using ELISA do not conduct this form of validation, resulting in misleading quantitative data on oxylipin levels in publications.

SPM are reported to be present in small amounts and to be challenging to analyze. For many researchers, detecting SPM using LC-MS/MS has proven extremely difficult or impossible, and the actual levels present in biological samples have been questioned<sup>12-13</sup>. The LC-MS/MS methods used for measuring SPM have been challenged. In part to address this uncertainty, broadly-based community guidelines have been recently published<sup>12-15</sup>. The discrepancies between ELISA and LC-MS/MS values makes the need for verification even more critical for SPM. In one recent study, ELISAs were used to demonstrate that RvD1 levels are significantly reduced in CSF of patients with Parkinson's disease, suggesting a role for this lipid in the pathology<sup>16</sup>. In that study, both RvD1 and RvD2 were reported in plasma and CSF around 120 or 80 pg/ml respectively, without verification using LC-MS/MS<sup>16</sup>. However, these plasma levels contrast with data from others showing that in fresh plasma, SPM are either absent or only detected after samples are left for extended periods at room temperature<sup>17,18</sup>. This accords with decades old studies showing appearance of non-enzymatically-derived isoprostanes in plasma during extended storage<sup>19</sup>. Similarly, a more recent study claimed high levels of maresin 1 (MaR1) and lipoxin A<sub>4</sub> (LXA<sub>4</sub>) in a mouse model of arthritis, using ELISA with no LC-MS/MS validation<sup>20</sup>.

Prompted by these contradictory data in the literature and concerns that SPM could potentially be non-enzymatically generated as artefacts, we compared ELISA with LC-MS/MS for the analysis of RvD1 and RvD2 in serum and plasma, both using published data and experimentally. Overall, we found that ELISA analysis overestimates levels of both oxylipins, including in samples where these lipids are not detected at all using LC-MS/MS, and that signals detected using ELISAs increase when sample processing isn't fully controlled, as recently shown for LC-MS/MS<sup>17</sup>.

## Methods

### *Literature review*

Values for the levels of resolvins in healthy humans were obtained from the original source materials included in a review by Calder summarising a large body of work prior to 2020<sup>21</sup>. Next, a PubMed search ([www.ncbi.nlm.nih.gov/pubmed/](http://www.ncbi.nlm.nih.gov/pubmed/), accessed in May 2025) identified studies published later than 2020, using the keywords "resolvin D1" or "resolvin D2" and "plasma" or "serum".

### *Extraction of lipids from plasma or serum.*

Pooled normal human plasma (Plasma 1) was from Alpha Laboratories, Hampshire, UK (<https://www.alphalabs.co.uk/ccn-15>). Pooled normal human serum (Serum 1) was from Merck Life Sciences, Dorset, UK (<https://www.sigmaaldrich.com/GB/en/product/sigma/h4522>). Blood from healthy volunteers was obtained at Cayman Chemical in accordance with the Declaration of Helsinki by the World Medical Association<sup>22</sup>. This blood was processed to form plasma or serum immediately, except as outlined below. To test the impact of sample handling and storage on the detection of SPM signals, blood from five volunteers was obtained and split in four aliquots each. From three of them, serum was obtained by incubating at room temperature for 1 h, followed by centrifugation at 1500 x g for 30 min. The supernatant was either immediately frozen (serum control) or incubated at 37 °C for 24 or 96 h prior to freezing. The fourth blood aliquot was incubated for 24 h at 37 °C, followed by centrifugation as described above and freezing of the supernatant. Samples were stored at -80 °C then used for ELISA and LC-MS/MS as outlined below. For the spike and recovery test, five aliquots of plasma (1 ml) were spiked with increasing amounts of authentic RvD1 (in pg): 0, 250, 500, 1000 and 2000. These samples were split in two for separate extraction and ELISA or LC-MS/MS analysis.

Prior to extraction, samples were thawed on ice, then vortexed, and the required volume for extraction was aspirated. Samples were processed to isolate oxylipins using either the solid phase extraction (SPE) protocols described in the ELISA kits, a slightly different SPE method recommended as being specifically optimised for SPM<sup>23-27</sup>, or an SPE method optimized by Cayman for a broad range of oxylipins, as detailed below.

(i) SPE protocol for RvD1 ELISA.

This was undertaken in line with the ELISA kit recommended instructions (Cayman Chemical). SPE cartridges (C18, 6 cc, 500 mg, Sep-Pak Vac, Waters) were conditioned with 5-10 ml HPLC grade methanol followed by 10 ml Ultrapure water. Sample aliquots (500 µl) were loaded and allowed to adsorb onto the SPE column. Each SPE cartridge was then washed with 10 ml Ultrapure water followed by elution using 3 ml ethyl acetate into 10 ml glass tubes. The eluate was dried using a RapidVap vacuum system (Labconco), or under a stream of nitrogen, then resolvated in ELISA buffer, vortexed and centrifuged (527 x g, 1 min, 4 °C) before adding 50 µl to the ELISA plate and following manufacturer's instructions for completion of the ELISA. Where LC-MS/MS analysis was performed, samples were resolvated in methanol.

(ii) SPE protocol for RvD2 ELISA.

This was undertaken in line with the ELISA kit recommended instructions (Cayman Chemical). Aliquots of plasma or serum (500 µl) were acidified with 500 µl 100 mM sodium acetate (pH adjusted to 5 with glacial acetic acid) and vortexed. SPE cartridges (C18, 6 cc, 500 mg, Sep-Pak Vac, Waters) were conditioned with 10 ml HPLC grade methanol followed by 10 ml Ultrapure water. The acidified sample (total 1 ml) was then loaded to the SPE cartridge and allowed to adsorb onto the column. Each cartridge was washed with 10 ml HPLC grade water then eluted using 3 ml HPLC grade methanol. The eluate was evaporated using vacuum evaporation or a stream of nitrogen, as for RvD1. The extract was reconstituted in ELISA buffer, vortexed and centrifuged (527g, 1 min, 4 °C) before adding 50 µl to the ELISA plate, and following manufacturer's instructions for completion of the ELISA. Where LC-MS/MS analysis was performed, samples were resolvated in methanol.

(iii) Optimized published SPE protocol for RvD1 and RvD2 analysis<sup>23-27</sup>.

Aliquots of plasma or serum (500 µl) were added to 15 ml polypropylene tubes. For plasma, 6 separate samples were processed, then combined post-extraction so that in total 3 ml of plasma underwent SPE. Ice-cold HPLC-grade methanol (2 ml) was added, and the tube capped, vortexed and incubated at -20 °C for 45 min, for protein precipitation. After centrifugation (1000 x g, 10 min, 4 °C) the supernatants were transferred using a glass pipette to polypropylene tubes and kept at 4 °C. Sample volumes were reduced under N<sub>2</sub> stream, then water (HPLC grade water, acidified to pH 3.5 using glacial acetic acid) was added in a 9:1 ratio. Samples were vortexed and placed on ice. SPE cartridges were conditioned using 10 ml HPLC-grade methanol followed by 10 ml HPLC-grade water. Then, 5 ml of the acidified sample was added to the SPE cartridge, allowed to adsorb onto and pass through at a gentle rate, followed by the remaining 5 ml sample. The column was then washed with 10 ml HPLC grade water, followed by 5 ml HPLC-grade hexane, and eluted using 8 ml methyl formate into glass tubes. Extracts were dried, first under a gentle stream of nitrogen, then to completion using a vacuum evaporator. For plasma, individual samples were pooled during the drying process. Lipid extracts were then resolvated into either ELISA buffer or methanol for RvD1 or RvD2 analysis using ELISA or LC-MS/MS. Following SPE and evaporation of solvent, samples were immediately reconstituted in the relevant ELISA buffer and then tested for immunoreactivity as described in the kit instructions, on the same day, in triplicate. For LC-MS/MS analysis, the dried plasma or serum extracts were resolvated into 100 or 50 µl methanol respectively, then stored at -80 °C (1-4 days) before analysis.

#### (iv) SPE protocol optimized by Cayman.

This protocol was used in the spike and recovery analysis. To 500 µl aliquots of plasma, 30 µl internal standard solution containing 50 pg/ml RvD1-d<sub>5</sub> in water/acetonitrile 6:4 (v/v) was added, and samples were placed at -80 °C for 1 h to improve extraction. After thawing, 470 µl acetonitrile was added. Tubes were vortexed and then centrifuged at 15000 x g for 15 min. The corresponding wells of a 96-well SPE plate (Strata-X 33 µm polymeric reversed phase, 60 mg) were conditioned with 1.75 ml methanol followed by 1.75 ml water, using a nitrogen-driven positive-pressure device from Biotage. Sample supernatants were diluted with 2 ml water prior to loading (2 x 1.7 ml) on the SPE plate. After washing with 5 x 1 ml water, extracts were eluted with 1.25 ml methanol into a 96-well collection plate and dried using a vacuum evaporator overnight. Extracts were reconstituted with 80 µl acetonitrile and transferred to glass autosampler vial inserts. The corresponding wells in the collection plate were washed with 120 µl water, which was added to the autosampler vial inserts before capping, vortexing and placing in the system autosampler for injection into the LC-MS/MS system.

#### *LC-MS/MS analysis.*

Lipids were separated using a Kinetex C18 column (2.6 µm, 100 Å, 100 x 2.1 mm, Phenomenex) on a Shimadzu Nexera UHPLC system coupled to a Sciex 6500 QTrap mass spectrometer. A binary gradient at 300 µl/min was used with mobile phase A (HPLC-grade water, 0.1 % formic acid) and mobile phase B (HPLC-grade acetonitrile, 0.1 % formic acid). Gradient was: 0 - 0.2 min, 40 % B; then from 0.2 - 4 min, a linear increase to 95 % B, held till 7 min. From 7 - 7.2 min, gradient was decreased to 40 % B, and held until 12 min. Column temp 25 °C, sample injection 10 µl. RvD1 and RvD2 were detected in negative-ion mode using multiple reaction monitoring (MRM) *m/z* 375.2/215.1, and 375.2/141.1, respectively. Settings for RvD1 were: DP, 35; CE, 24; CXP, 23. Settings for RvD2 were: DP, 15; CE, 22; CXP, 15. Dwell time was 50 ms, and ion source parameters were: GS1, 60 psi; GS2, 60 psi; curtain gas, 35 psi; ion spray voltage, -4500 V; and temperature, 600 °C. Data was analysed using Analyst 1.7 (Sciex). Limit of quantitation (LOQ) was down to around 2.5 pg on column at signal:noise ratio 5:1 for RvD1 and RvD2 calibration standards (both from Cayman Chemical).

The above system was used at Cardiff while parallel analysis at Cayman used the same method with modifications as follows: an Exion AC HPLC system coupled to a Sciex 6500+ QTrap mass spectrometer. A binary gradient at 300 µl/min with mobile phase A (HPLC-grade water, 0.1 % formic acid) and mobile phase B (HPLC-grade acetonitrile, 0.1 % formic acid). Gradient was: 0 - 0.2 min, 40 % B; 0.2 - 3 min, linear increase to 80 % B; 3-6.5 min, linear increase to 95 % B, held till 11.5 min, 11.5-11.6 min, decrease to 40 % B, held until 15 min. Column temp 25 °C, sample injection 20 µl. RvD1 and RvD2 were detected using MRM transitions 375.2/141.1, and 375.2/175.1, respectively. Settings were: DP, 100; CE, 25; CXP, 10; dwell time, 10 ms; GS1, 35 psi; GS2, 35 psi; curtain gas, 35 psi; ion spray voltage, -4500 V; and temperature, 450 °C. Limit of quantitation (LOQ) was around 5 pg on column at signal:noise ratio 5:1 for RvD1 and RvD2 calibration standards (both from Cayman Chemical). Due to the structural similarity of RvD1 and RvD2, both can be detected in MRM channels 375.2/215.1, and 375.2/141.1, while 375.2/175.1 is unique for RvD2. They are distinguished on LC-MS/MS by their retention times with RvD1 eluting around 0.2 min later than RvD2.

## **Results and Discussion**

*Statistical analysis of literature values for RvD1 and RvD2 in plasma and serum show they are significantly higher using ELISA than LC-MS/MS.*

First, we compared values reported in the literature for two SPM, measured using either ELISA or LC-MS/MS. A total of 52 studies were identified that contained serum or plasma values for RvD1 or RvD2 in healthy individuals, with around 60 % using LC-MS/MS (Supplementary Tables 1-4). Only one study performed a direct comparison of ELISA with LC-MS/MS on the same samples<sup>28</sup>. For serum, RvD1 levels ranged from 41.5 to 6750 pg/ml, with a mean value of 1609 pg/ml, using ELISA, in contrast to 0.4 to 400 pg/ml, with a mean of 111.6 pg/ml, using LC-MS/MS (Figure 1 A). Too few values were available for a meaningful comparison of RvD2 values using both methods in serum. Plasma RvD1 levels using ELISA varied from around 6 to more than 50000 pg/ml, with a mean of 4971 pg/ml, while LC-MS/MS showed 0 to 359 pg/ml, with a mean of 39 pg/ml (Figure 1 B). Plasma RvD2 was reported using ELISA from around 6 to 887 pg/ml, with a mean of 250 pg/ml, while LC-MS/MS reported from 0 to 699 pg/ml with a mean of 52.4 pg/ml (Figure 1 C). Overall, there is a larger variability and higher values reported with ELISA compared to LC-MS/MS (Figure 1), with mean values being 14.4-fold (serum RvD1), and 127-fold (plasma RvD1) and 4.8-fold (plasma RvD2), higher for ELISA than for LC-MS/MS.

While some studies detected RvD1 and RvD2 in plasma from healthy control subjects using LC-MS/MS, others reported a failure to detect these lipids (Supplementary Table 3). There are several possible reasons. First, it was demonstrated recently that storage of fresh plasma at room temperature for a period of hours leads to formation of signals corresponding to SPM, due to autoxidation<sup>17</sup>. Second, several studies that reported detectable RvD1 or RvD2 in plasma or serum described analytical methods and criteria that differ from established practices for MS detection of biomolecules<sup>15,29-38</sup>. A potential confounder is that healthy control samples across different studies may differ demographically. However, this seems an unlikely explanation, as the one study directly comparing the two methods showed around 10-fold higher levels of RvD1 measured by ELISA than the levels measured by LC-MS/MS in the same samples<sup>28</sup>. To assess this further, we next performed a direct comparison using both ELISA and LC-MS/MS methods on the same serum and plasma samples, also including extended incubations to assess the impact on measured SPM values.

#### *Experimental comparison of ELISA and LC-MS/MS to determine RvD1 and RvD2 levels in commercially-sourced human plasma and serum.*

A direct comparison of ELISA and LC-MS/MS was conducted, measuring RvD1 and RvD2 in the same human serum and plasma samples. For both lipids, sample preparation compared different SPE protocols, described above, optimized either by Cayman (methods (i) or (ii)) or academic laboratories working on SPM detection using LC-MS/MS (method (iii))<sup>23-27</sup>. Following SPE extraction, samples were run on ELISA or LC-MS/MS in parallel. An initial comparison used commercially-sourced serum and plasma (serum1, plasma1). Using ELISA, with samples prepared using either the ELISA kit SPE methods (i) and (ii) or the published SPE extraction method (iii), RvD1 and RvD2 immunoreactivity was reported in both serum and plasma, with higher levels observed in serum (Table 1). For plasma, the ELISA kit SPE methods (i) and (ii) recovered slightly more RvD1 and somewhat less RvD2 than the published SPM method (iii) (Table 1). ELISA SPE protocols had been separately optimized for each kit, so subtle differences when compared to the SPM optimised method are expected. However, with serum, the LC-MS-optimized method (iii) recovered much lower levels than the ELISA-optimized methods (i) and (ii), particularly for RvD2 (Table 1). With these commercially obtained samples, serum demonstrated far higher signals in comparison to plasma, especially when tested using the ELISA kits.

Next, LC-MS/MS analysis of lipids from the commercially-sourced plasma and serum, processed using the same SPE methods used for ELISA were conducted. Here, instead of reconstitution into ELISA buffer, lipids were resoluted into a small volume of methanol. For reference, the detection

of 5 pg RvD1 and RvD2 standards on column is shown (Figure 2 A,B). Here, the signal:noise ratios are >10, well above those commonly used to define LOQ (5) or LOD (3). Using the ELISA data as a guide, sufficient plasma or serum was processed using all three SPE methods to generate extracts that should result in a clearly visible peak when injecting 10 or 20 % of the total sample volume on column. For plasma, 3 ml was processed by extracting 6 aliquots of 500 µl plasma each before pooling the eluates into a single sample which was dried and reconstituted into 100 µl methanol, from which, 10 µl was analysed. For serum, where larger signals were detected on ELISA, a 500 µl sample was extracted, dried, then reconstituted into 50 µl before 10 µl injection. Details of pg amounts on column expected for each of these samples (based on ELISA data) are in Supplementary Table 5. For most samples, only baseline noise was observed in chromatograms. Specifically, RvD1 or RvD2 signals were undetected in the commercially-sourced plasma processed using methods (i),(ii) or (iii) (Figure 2 C-F), while RvD2 was not detected in the commercially-sourced serum (Figure 3 A,B).

For RvD1 in serum, very noisy peaks eluting close to the retention time for the RvD1 standard were observed, all showing signal:noise ratios below those required to define the LOD (Figure 3 C,D). Notably, they did not accord with what was expected based on the ELISA data (132-661 pg on column) where large peaks should have been visible. Next, we obtained enhanced product ion (EPI) spectra of the  $m/z$  375.2 ion at the retention time of these weak signals, where RvD1 should elute, and compared these with the RvD1 standard as published previously, which shows prominent ions at  $m/z$  141, 215, and 233<sup>39</sup>. However, our spectrum did not compare well, with few expected ions apparent (Fig. 3 E,F). These data show that these weak signals do not correspond to actual RvD1 present in these serum samples, and that it is not possible to generate MS/MS spectra to aid structural confirmation in the absence of a definable chromatographic peak.

#### *Further analysis using freshly obtained serum and plasma, and the impact of storage conditions on ELISA detection of SPM.*

As the previous comparison was performed using single commercially-sourced samples, we next generated plasma and serum from blood freshly obtained from three workplace volunteers at Cayman. This allowed us to compare ELISA and LC-MS/MS detection of SPM in plasma or serum which was known to have not been subjected to any extended or improper storage conditions, which has been shown to lead to artefactual generation of oxylipin signals, including SPM and isoprostanes<sup>17,19</sup>. Using ELISA, detectable signals were obtained in all samples, with an expected degree of individual variation amongst donors observed (Table 2). However, none of these extracts showed detectable RvD1 or RvD2 chromatographic signals when analyzed using LC-MS/MS. This demonstrates that freshly processed plasma or serum can generate positive signals in ELISA assays for SPM.

Next, the impact of various processing conditions on these signals was tested using freshly obtained blood from five volunteers. For this experiment, a slightly modified Cayman in-house SPE extraction method (iv) optimized for a wide variety of oxylipins was used for the LC-MS/MS samples. Using ELISA, reported SPM levels in freshly prepared serum were low. However, both oxylipins were readily detected at significantly higher levels, when serum was incubated for 24 or 96 h at 37 °C, or when prepared after incubating the blood at 37 °C for 24 h (Figure 4). As before, no signals of either SPM were detected in any of these samples using LC-MS/MS. These results suggest that poorly controlled handling of blood in the preparation of serum can result in artefactual generation of substances that show immunoreactivities mimicking but not reflecting actual RvD1 or RvD2.

#### *Spike and recovery of RvD1 in plasma.*

Last, a spike-and-recovery experiment was conducted by adding varying amounts of RvD1 to plasma prior to extraction, then comparing values reported using either LC-MS/MS or ELISA and calculating recovery rates. For ELISA, recovery rate was overall 77 % (Table 3). Similarly, for LC-MS/MS, the recovery of RvD1 was 83 %. This shows that both methods can detect spiked SPM equally well, ruling out any technical problems with the detection of authentic SPM when present in a complex tissue matrix.

## Conclusion

In summary, ELISA analysis of serum and plasma can report levels of RvD1 and RvD2 that are not verifiable using LC-MS/MS, especially with samples that are not handled carefully. This strongly reinforces the need stated in manufacturer's protocols for proper handling of blood and any other biological samples and, to use LC-MS/MS to verify the quantitative data generated using ELISA. Manufacturers often provide cross reactivity data on dozens of species, but it is generally not possible to characterize cross reactivity fully against every similar molecule. Although we only tested one kit for each SPM, the same issues identified here are expected to apply to other commercially-available oxylipin ELISAs. Further work to ascertain cross reactivity of ELISA antibodies with serum components will continue to be undertaken to support the best use of this technology, and users are strongly advised to follow the manufacturers' guidance when reporting data relating to SPM analysis. Our LC-MS/MS data showing an absence of RvD1 and RvD2 signals in extracted plasma is in line with other reports which also failed to detect these lipids in plasma<sup>17,18</sup>.

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**COI statement.** The authors declare no conflicts of interest.

## Figure Legends

**Figure 1. Statistical analysis of published literature shows ELISAs report higher levels than LC-MS/MS.** Summarized data from studies using either ELISA or LC-MS/MS to analyze serum (A) or plasma (B,C), from the data listed in Supplementary Tables 1-4. Data from healthy control subjects were obtained from source publications and are shown as box and whisker plots (median, IQR, range, outliers shown). In Panel B, inset shows spread of values, without outlier plotted.

**Figure 2. LC-MS/MS chromatograms of RvD1 and RvD2 standards and of human plasma, showing the lack of detection of the expected lipid signals.** Panels A,B. Multiple reaction monitoring (MRM) chromatograms of 5pg of RvD1 and RvD2. RvD1, eluting at 1.98 min, RvD2, eluting at 1.81 min, analysed as in Supplementary Methods. Panel C. Plasma processed using the RvD1 ELISA SPE method (i) and analysed for RvD1. Panel D. Plasma processed using the RvD2 ELISA SPE method (ii) and analysed for RvD2. Panels E,F. Plasma processed using SPE method (iii) and analysed for RvD1 or RvD2, as shown. Amounts expected retention time for standards, and MRM transitions used are shown on figures.

**Figure 3. LC-MS/MS chromatograms for serum for RvD1 and RvD2, and enhanced product ion spectra from serum extracts, for  $m/z$  375.2 precursor ions obtained at 1.9 min for RvD1.** Panel



A. LC-MS/MS of extracted serum processed using SPE method (iii) for RvD2. Panel B. LC-MS/MS of extracted serum processed using SPE method (ii) for RvD2. Panel C. LC-MS/MS of extracted serum processed using SPE method (iii) for RvD1. Panel D. LC-MS/MS of extracted serum processed using SPE method (i) for RvD1. Amounts of RvD1 or RvD2 expected retention time for standards and MRM transitions used are shown on figures. Panels E,F. Enhanced product ion spectra obtained at the expected retention time for RvD1, during elution of serum lipids, are shown.

**Figure 4. Storage of blood and serum leads to immunoreactive SPM-like lipids being detected by ELISA.** Samples were generated and processed as indicated in Methods, and analyzed using ELISA for RvD1 (Panel A) and RvD2 (Panel B). n=5 donors, mean +/- SEM. One way ANOVA with Tukey Post Hoc Test, \*\* P< 0.01.

## Tables

**Table 1. ELISA analysis data for RvD1 and RvD2 in commercially sourced plasma and serum.** Samples were extracted and analyzed in triplicate as described in Methods. Mean values shown.

Sample	SPE protocol	RvD1 (pg/ml)
Plasma 1	(i)	39.1
Plasma 1	(iii)	30.2
Serum 1	(i)	6641
Serum 1	(iii)	1384
Sample	SPE protocol	RvD2 (pg/ml)
Plasma 1	(ii)	73.4
Plasma 1	(iii)	122.6
Serum 1	(ii)	5884
Serum 1	(iii)	540.8

**Table 2. ELISA data from freshly generated serum and plasma for RvD1 and RvD1** Several dilutions of each sample were analyzed as described in Methods. Mean value shown.

Sample	SPE protocol	RvD1 (pg/mL)
Plasma 2	(i)	29.3
Plasma 3	(i)	166
Plasma 4	(i)	97.0
Serum 2	(i)	24.7
Serum 3	(i)	69.5
Serum 4	(i)	75.2
Sample	SPE protocol	RvD2 (pg/mL)
Plasma 2	(ii)	84.0
Plasma 3	(ii)	47.5
Plasma 4	(ii)	85.6

**Table 3. Spike and recovery of RvD1 added to plasma and measured using either LC-MS/MS or ELISA.** RvD1 and RvD1-d<sub>5</sub> were added to plasma and extracted and analyzed as indicated in Methods.

Spiked in 0.5 ml plasma (pg)	Measured by ELISA (pg)	Measured by LC-MS/MS (pg)
0	4.1	<LOD
125	75	110
250	223	215
500	417	392
1000	742	792

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## APPENDIX 1

**Supplementary Table 1. Published reports of serum RvD1 and RvD2 in the literature, measured by LC-MS/MS.**

SPM	Concentration (pg/ml)	Subjects	Reference
RvD1 RvD2	200 900	19 healthy controls, 37% male	39
RvD1	64.7	3 healthy controls, mean 60 yr	28
RvD1 RvD2	0.4 0.3	83 healthy controls, mean 40 yr Figure states n = 5 measured	29
RvD1	1.8	7 healthy controls; mean ~42 yr, 71% male Detected in one individual	31
RvD1	2.7	3 healthy participants, mean 25 yr	30
RvD1 RvD2	400 2200	3 healthy controls, mean 30 yr	40

**Supplementary Table 2. Published reports of serum RvD1 or RvD2 in the literature, measured by ELISA.**

SPM	Concentration (pg/ml)	Subjects	Reference
RvD1	564	16 healthy participants, mean 55 yr, 93.8% male	41
RvD2	255	100 healthy controls, 19-79 yr	42
RvD1	1249	49 healthy controls	43
RvD1	290	30 healthy adolescents	44
RvD1	1470	123 healthy controls	45
RvD1	2000	135 healthy controls	46
RvD1	41.5	20 healthy controls	47
RvD1	6750	52 healthy controls, mean 55 yr	48
RvD1	694	27 healthy controls	49

**Supplementary Table 3. Published reports of plasma RvD1 and RvD2 in the literature, measured by LC/MS/MS**

SPM	Concentration (pg/ml)	Subjects	Reference
RvD1 RvD2	3.7 2.8	10 healthy participants, age 34 yr, 60% male	32
RvD1 RvD2	38 66.4	21 healthy participants, age 54 yr	50
RvD1 RvD2	62.1 44.6	Infants at birth (umbilical cord plasma)	51
RvD1 RvD2	61.2 37.4	Infants; age 6 mo	51
RvD1 RvD2	44 37.5	Children; age 5 yr	51
RvD1 RvD2	67.5 33.4	43 infants at birth (umbilical cord plasma)	52
RvD1 RvD2	53 30.4	19 Children, age 12 yr	52
RvD1	3.5	32 controls, details not given	53
RvD1	2.3	7 healthy controls; age ~42 yr, 71% male	31
RvD1 RvD2	71 53	8 healthy participants, age 31 yr, 37.5% male	54
RvD1 RvD2	61 6.4	138 infants at birth (umbilical cord blood)	55
RvD1	0.19	14 healthy subjects, age 36 yr, 43% male	33
RvD1 RvD2	0.21 0.78	22 healthy participants, age 26 yr, 41% male	35
RvD1 RvD2	6.39 0.6	10 healthy participants, age 54.4 yr, 40% male	34
RvD1	0	6 healthy volunteers	18
RvD1	0	121 healthy males and females	2
RvD1 RvD2	359 699	20 healthy controls	56
RvD1 RvD2	38 66	21 men and women undergoing elective joint replacement, age 53	57
RvD1 RvD2	42 27	17 aged 51, breast surgery 15 aged 44, laparoscopy	58
RvD1 RvD2	0 0	10 healthy controls	1
RvD1 RvD2	120 67	21 (10 m, 11 f) aged 20–70 yr	59
RvD1 RvD2	38 32	35 Women with obesity	60
RvD1 RvD2	51 32	22 male volunteers, 38-62 yr	61

RvD1	0	Healthy controls	62
RvD2	0		
RvD1	0	10 healthy volunteers	36
RvD2	2.3		
RvD1	1.48	20 patients with ALS	37
RvD2	1.23		
RvD1	7.4-8.7	479 men, 499 women (detected in 18 m, 27 f)	63
RvD2	6.4-6.6	479 men, 499 women (detected in 40 m, 28 f)	
RvD1	~8	10 out of 35 healthy controls measured	27
RvD2	~10		
RvD1	~0.65	23 healthy adults, 18-45 yr	64
RvD2	~0.8		

**Supplementary Table 4. Published reports of plasma RvD1 and RvD2 in the literature, measured by ELISA.**

SPM	Concentration (pg/ml)	Subjects	Reference
RvD1	65.1	30 controls, mean 50.5 yr 3.3% male	65
RvD1	2617	136 pregnant women at delivery, mean 28.9 yr	55
RvD2	819		
RvD1	~6	26 healthy individuals, mean 33.8 yr, 42% male	66
RvD2	~6		
RvD1	218	39 healthy controls, mean 26.6 yr	67
RvD1	50118	17 healthy pregnant women	68
RvD1	310	6 healthy non-smokers, 18-25 yr, 50% male	69
RvD2	887		
RvD1	61	138 infants at birth (umbilical cord blood)	55
RvD2	6.4		
RvD1	124	7 healthy controls	16
RvD2	83	8 healthy controls	
RvD1	10147	20 healthy donors	70
RvD1	283-359	60 participants with ulcerative colitis	71
RvD1	~220	20 healthy participants, mean 60 yr, 50% male	72
RvD1	10	11 healthy women, mean 48.6 yr	73
RvD2	8		
RvD2	~100	18 out of 35 healthy controls measured	27
RvD1	~400	20 healthy controls, mean 66.5 yr	74
RvD2	90	2633 mixed population subjects	75



**Supplementary Table 5. Volumes of plasma and serum extracted for LC/MS/MS analysis.**  
Expected pg on column is calculated based on ELISA data.

No.	Sample type	SPE method	Lipid	Sample extracted (ml)	Resuspended volume (μl)	Injection volume (μl)	pg expected on column
1	Plasma1	(i)	RvD1	3	100	10	11.7
2	Plasma1	(ii)	RvD2	3	100	10	22.0
3	Plasma1	(iii)	RvD1	3	100	10	9.0
4	Plasma1	(iii)	RvD2	3	100	10	36.8
5	Serum1	(i)	RvD1	0.5	50	10	664.1
6	Serum1	(ii)	RvD2	0.5	50	10	588.4
7	Serum1	(iii)	RvD1	0.5	50	10	132.1
8	Serum1	(iii)	RvD2	0.5	50	10	54.1

Figure 1

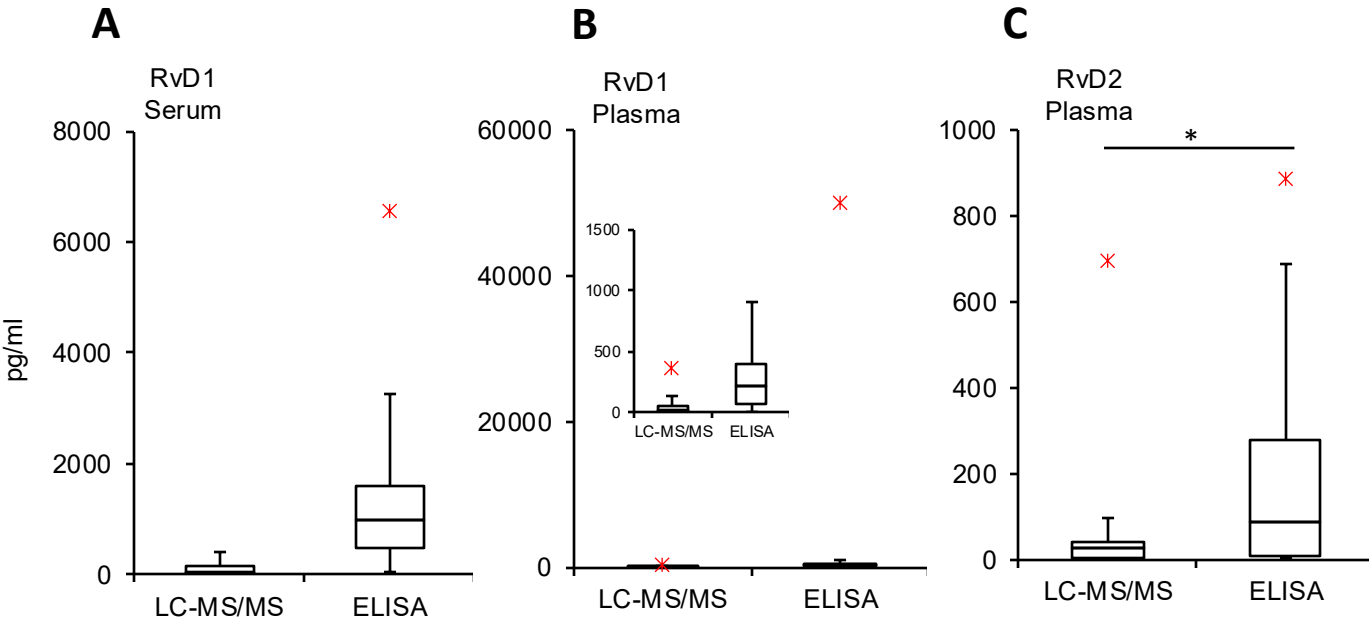
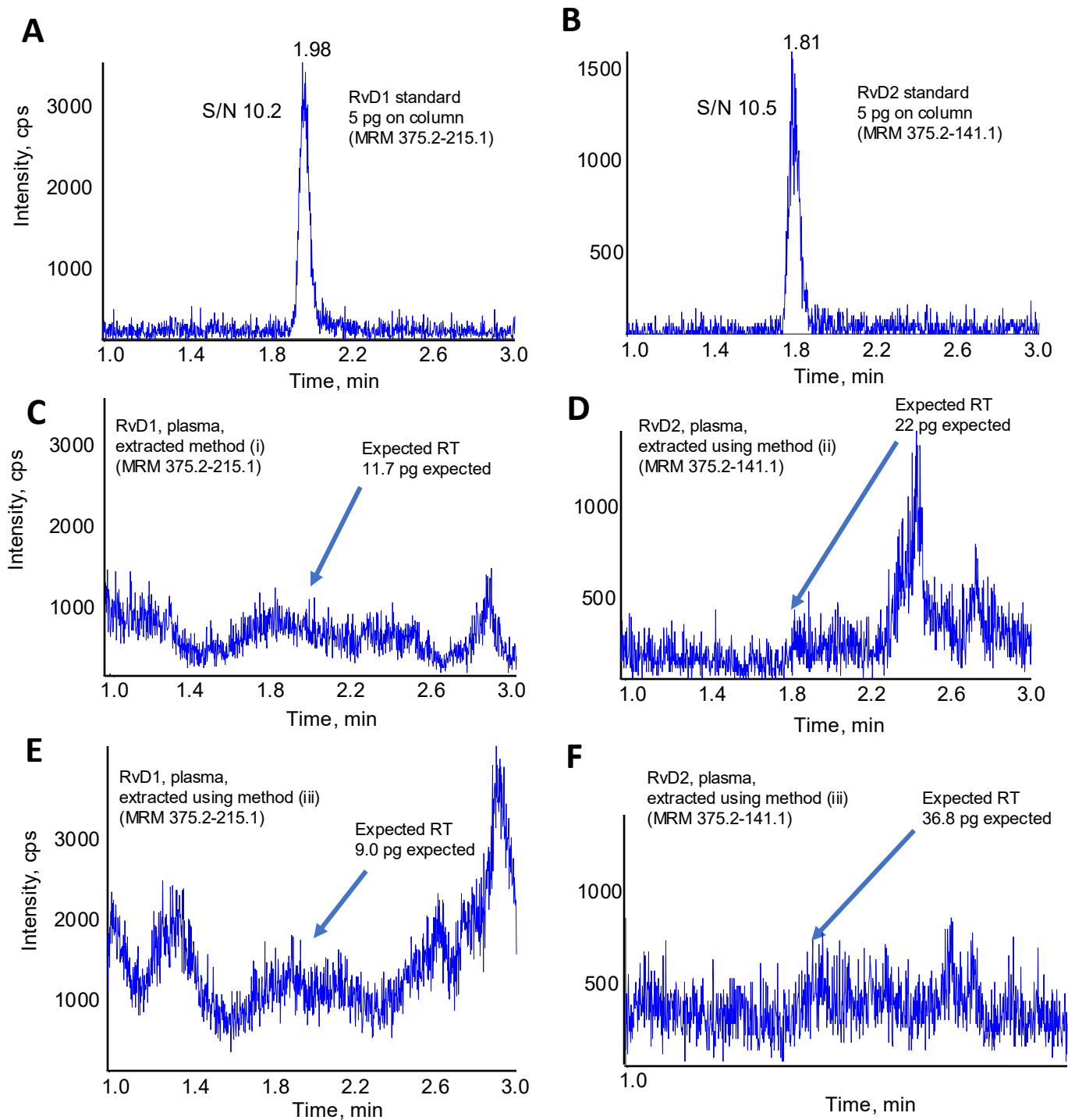


Figure 2



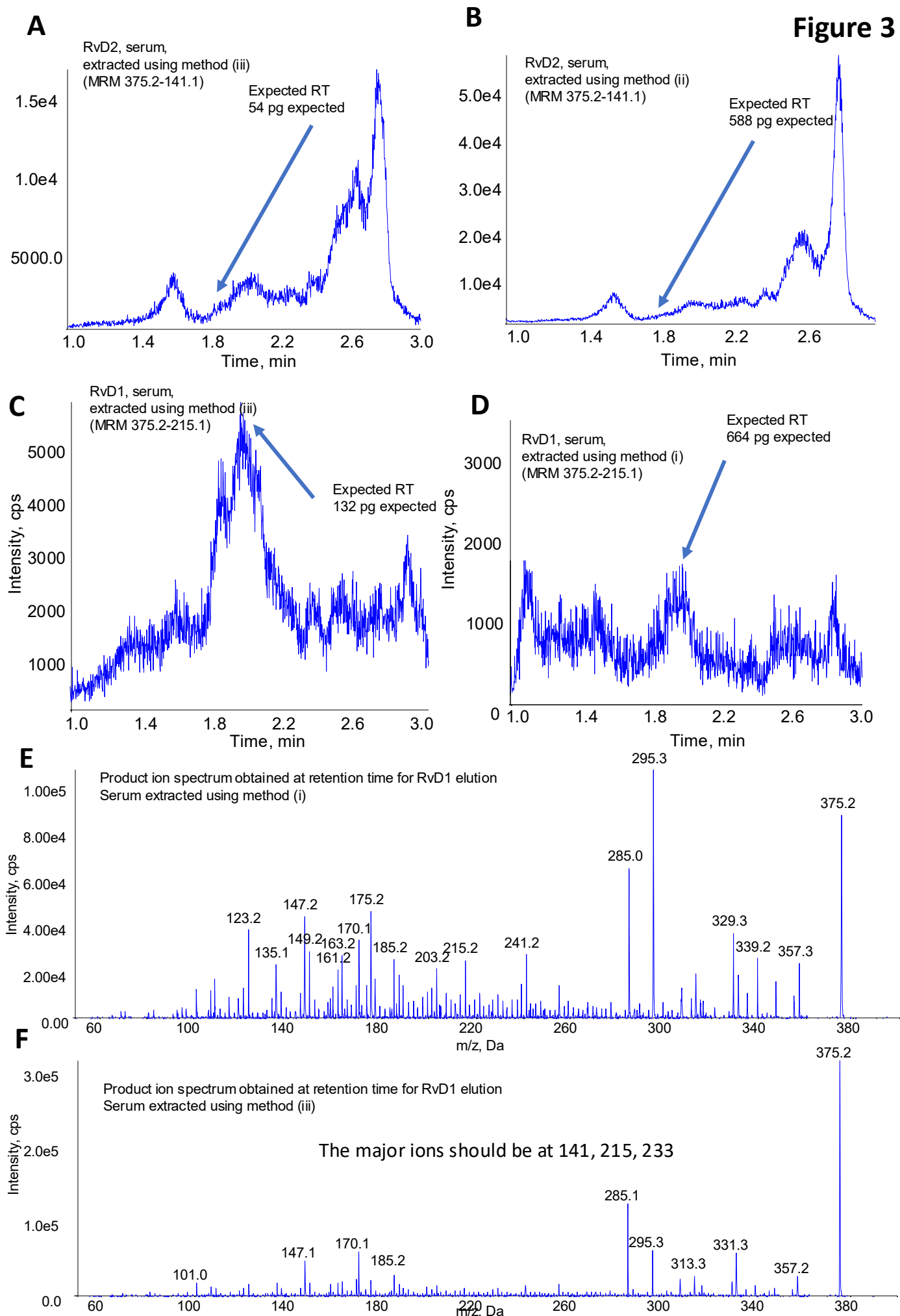
**Figure 3**

Figure 4

