Journal of Extracellular Vesicles

ISEV

PERSPECTIVE OPEN ACCESS

Quantitative Lipid Analysis of Extracellular Vesicle Preparations: A Perspective

Tore Skotland[1,2] | Kim Ekroos[3] | Alicia Llorente[1,2,4] | Kirsten Sandvig[1,2,5]

[1]Department of Molecular Cell Biology, Institute for Cancer Research, Oslo University Hospital, The Norwegian Radium Hospital, Oslo, Norway | [2]Centre for Cancer Reprogramming, Faculty of Medicine, University of Oslo, Oslo, Norway | [3]Lipidomic Consulting Ltd., Espoo, Finland | [4]Department for Mechanical, Electronics and Chemical Engineering, Oslo Metropolitan University, Oslo, Norway | [5]Department of Biosciences, University of Oslo, Oslo, Norway

Correspondence: Tore Skotland (toresko@uio.no)

Received: 22 October 2024 | **Accepted:** 5 February 2025

Keywords: extracellular vesicles | exosomes | lipid analyses | mass spectrometry | membranes

ABSTRACT

Quantitative lipidomic analysis performed by mass spectrometry is required for determination of the lipid content of extracellular vesicles (EVs). Such methods can provide information about the total amount of lipids, the lipid species composition, the purity of EV samples as well as the cellular origin of the EVs. There are, however, many pitfalls when performing lipid analyses. Thus, any non-specialist should collaborate with experts in lipidomics. In addition to many good review articles giving advice about lipid analyses, we recommend the information and guidelines published by the Lipidomic Standard Initiative, an interest group affiliated with the International Lipidomics Society.

1 | Introduction

Extracellular vesicles (EVs) are a heterologous group of vesicles released from cells. They consist of a lipid bilayer surrounding a liquid phase and contain proteins, nucleic acids, and various types of metabolites. EVs can originate both from intracellular organelles and from the plasma membrane (Welsh et al. 2024). Most focus on EVs of intracellular origin has been on exosomes, which originate from the intraluminal vesicles (ILVs) in the multivesicular bodies (MVBs) and are released after fusion of MVBs with the plasma membrane (PM) (Skotland et al. 2020). EVs of intracellular origin can also be excreted as a result of secretory autophagy (Khaldoun et al. 2014; Hessvik et al. 2016; Ariotti et al. 2020). The EVs released from the plasma membrane are often referred to as ectosomes; see van Niel et al. (2022) for an overview. These EVs include vesicles formed by various types of cell death mechanisms; see Skotland and Sandvig (2022) and references therein.

The most common membrane lipids (with exception of cholesterol) are listed in Table 1, and structures of some lipid species are shown in Figure 1. Mammalian membranes have an asymmetric lipid distribution in the two leaflets making up the membrane bilayer. The PM has most (if not all) sphingolipids and phosphatidylcholine (PC) localized in the outer leaflet, and most (if not all) other phospholipids, for example, phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI), in the inner leaflet (van Meer et al. 2008). The lipid distribution in the bilayer of exosomes can at the time of their release by healthy cells be expected to closely resemble that of the PM. However, little is known about whether, or to which extent, this asymmetry of the exosome bilayer is changed during storage or freezing/thawing; see discussion about asymmetry in Skotland et al. (2020). It is established that EVs released from the PM by several death mechanisms will have at least some PS in the outer leaflet (Skotland and Sandvig 2022; Atkin-Smith et al. 2017; Zargarian et al. 2017); see illustration in Figure 2. PS localization is important for the fate of these EVs since exposed PS on the surface is expected to cause a rapid clearance by macrophages (Nagata 2018). Several authors write that the high content of PS in EVs will lead to a rapid uptake

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

© 2025 The Author(s). *Journal of Extracellular Vesicles* published by Wiley Periodicals LLC on behalf of International Society for Extracellular Vesicles.

TABLE 1 | The most common lipid classes in mammalian membranes. The table shows the names and common abbreviations for the head groups bound to the glycerol unit of phospholipids and to ceramide in sphingolipids.

Lipid classes/Abbreviations	*sn-1*	*sn-2*	Headgroups
Phosphatidylcholine/PC	FA[a]	FA	Choline
Lysophosphatidylcholine/lysoPC[b]	FA	OH	
Ether-linked PC (PC O- or PC P-)[c]	Alkyl or alkenyl	FA	
Phosphatidylserine/PS	FA	FA	Serine
Phosphatidylethanolamine/PE	FA	FA	Ethanolamine
Phosphatidylinositol/PI[d]	FA	FA	Inositol
Phosphatidylglycerol/PG	FA	FA	Glycerol
Phosphatidic acid/PA	FA	FA	H
Ceramides and sphingolipids do not contain *sn-1* and *sn-2* groups, but a long-chain sphingoid base (LCB) and an N-amidated FA as shown for SM d18:1/24:0 in Figure 1.			
Ceramide/Cer	LCB + FA		H
Sphingomyelin/SM	LCB + FA		Phosphocholine
Glycosphingolipids/GSLs[e]	LCB + FA		Carbohydrates

[a]FA = fatty acyl group.

[b]Lysolipids contain only one FA group and are common in most lipid classes, but for simplicity it is shown for PC only; most often the single FA group is present in the *sn-1* position.

[c]Ether-linked lipids occur in several lipid classes, but are for simplicity shown for PC only; most often the ether group (alkyl or alkenyl) is present in the *sn-1* position.

[d]PI can be phosphorylated at three sites giving rise to seven different forms called PIPs; for details see (Balla 2013).

[e]The glycosphingolipids classes have large variation in their carbohydrate structures; for details see (Merrill 2011).

by macrophages, but this is not the case if PS is present in the inner leaflet. Regarding this discussion it is interesting that some very small EVs (diameter of 30–40 nm) having caveolin on their surface were recently reported to be excreted from cells via secretory autophagy (Ariotti et al. 2020), thus indicating a bilayer structure opposite that of exosomes.

To use EVs (with or without drug) for therapeutic purposes, large-scale production will be necessary. Researchers have applied different forms of stress to cells to increase production of vesicles; reviewed in Antimisiaris et al. (2018). However, methods such as extrusion or sonication of cells can be expected to result in inversion of the lipid bilayer, or parts of it, thus affecting both the surface properties and making it more difficult to produce reproducible batches. It should also be noted that attempts to make liposomes with a lipid composition similar to that of the total EV composition will result in vesicles with a symmetrical lipid distribution, and thus a surface different from the EVs one wants to mimic.

In spite of new methods to separate different populations of EVs, many EV preparations are likely to contain a mixture of various forms of EVs. In addition, EV preparations may contain non-vesicular lipid-containing particles, such as exomeres (diameter of approx. 35 nm) released from cells (Zhang et al. 2018) and lipoproteins from plasma or serum in cell culture media. Lipoproteins are likely to copurify with various types of EVs due to their similar size and density; see discussion about purification of EVs and other preanalytical issues in Skotland et al. (2020). Partly due to this complexity of EV preparations, a group of scientists (International Society for Extracellular Vesicles; ISEV) has written guidance and recommendations for how to study EVs and characterize EV preparations. Their efforts have resulted in an initial short article published in 2014 (Lötvall et al. 2014), followed by two extensive articles describing what should be the Minimal Information for Studies of Extracellular Vesicles (MISEV), that is, MISEV2018 (Thery et al. 2018) and MISEV2023 (Welsh et al. 2024); the last with more than 500 references. These guidelines contain a lot of detailed and useful information. The chapter called quantification of total lipids should, however, be improved. In the present article, we first comment on the methods proposed for quantification of total lipids in these guidelines (Welsh et al. 2024; Thery et al. 2018). We then discuss how lipid analyses of EVs should be performed, and the knowledge we can obtain from these analyses.

2 | Methods for Quantification of Total Lipids Described in the MISEV Guidelines

Three methods for quantification of lipids are described in the two last MISEV guidelines, that is, the use of fluorescent intercalating dyes, total reflection Fourier-transform infrared (FITR) spectroscopy, and the sulfo-phospho-vanillin assay (Welsh et al. 2024; Thery et al. 2018). Here, we discuss shortly why these methods are not suitable to quantify the total lipids in EVs. Intercalating fluorescent dyes have for many years been used to study membrane properties since they can discriminate between membrane domains with differences in lipid order. However, since their fluorescence depends on local membrane properties and the dyes may also distribute differently among cellular membranes (Sezgin et al. 2014), they are not suitable to quantify total lipids of biological samples. Concerning the FITR method, it has been reported that this method can be used to

20013078, 2025, 3, Downloaded from https://isevjournals.onlinelibrary.wiley.com/doi/10.1002/jev2.70049 by Norwegian Institute Of Public Healt Invoice Receipt DFO, Wiley Online Library on [17/03/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

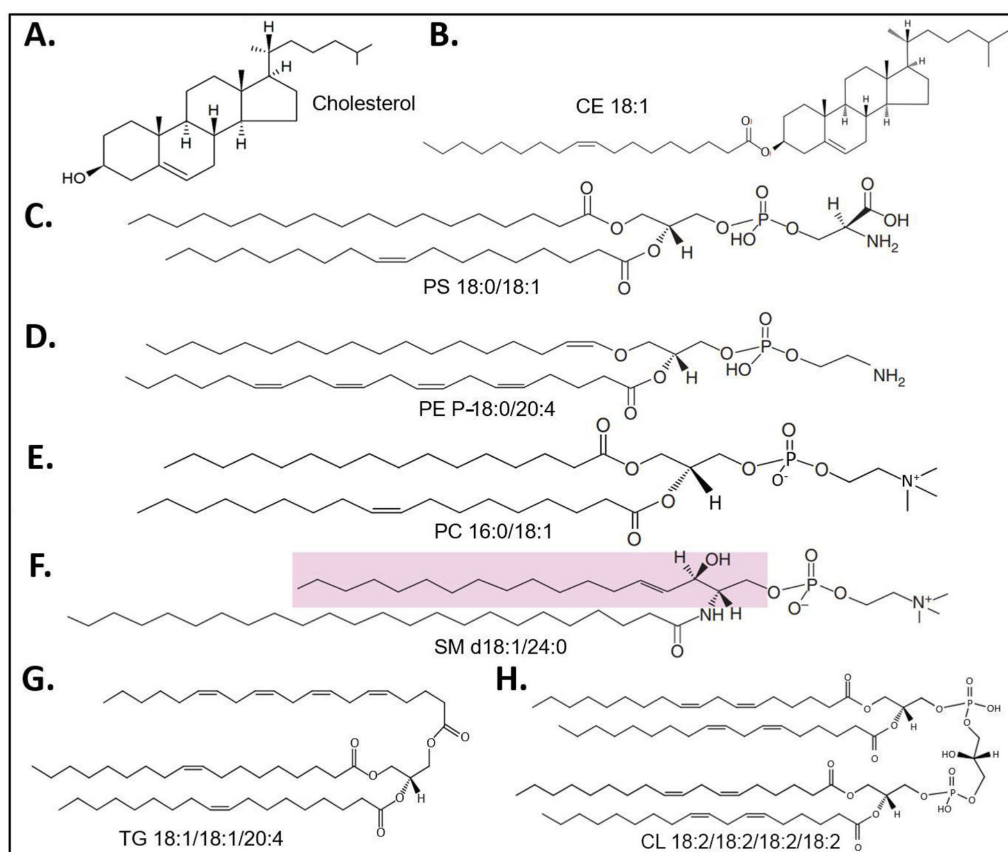

FIGURE 1 | Structures of some of the most common lipids in EV membranes. A. Cholesterol, the most common lipid molecule in probably all mammalian EV membranes. B. Cholesteryl ester CE 18:1. C. The phospholipid PS 18:0/18:1 is among the most common phospholipids in EVs, and illustrates two common features for phospholipids. They often contain a saturated lipid in the *sn-1* position and an unsaturated lipid (double bond in cis configuration) in the *sn-2* position. D. The ether-linked phospholipid PE P-18:0/20:4 has an alkenyl group in the *sn-1* position. Alkenyl-linked phospholipids are often called plasmalogens and PE plasmalogens are common in EVs. Ether-linked lipids may also contain an alkyl group lacking the double bond close to the headgroup. The species shown contain the very common polyunsaturated FA arachidonic acid (FA 20:4) in the *sn-2* position. E. The phospholipid PC 16:0/18:1, which is one of the most common lipid species in cells. F. Structure of the sphingolipid SM d18:1/24:0 with the long chain base (LCB) highlighted in red. Note that the N-amidated fatty acyl groups of sphingolipids may be much longer than the fatty acyl groups of phospholipids and may thus penetrate into the opposing leaflet. G. TG 18:1/18:1/20:4. H. Cardiolipin is the only known dimeric phospholipid; here we show CL 18:2/18:2/18:2/18:2. Cardiolipin is normally present only on the inner mitochondrial membrane. The structures shown for FA18:1 is for oleic acid, and the structures shown for FA 18:2 is for linoleic acid. The names shown for the lipids in this figure are given as 'short names' to make it easier to read; see Lipid Maps for the formally correct names, which include the positions of the double bonds. This figure was generated by using the structure drawing tools at Lipid Maps (https://www.lipidmaps.org/).

detect differences in spectra obtained for different EV populations (Mihály et al. 2017), but to our knowledge there is no publication demonstrating that this technology can be used to measure total lipid content. Of the three methods mentioned in the MISEV guidelines only the sulfo-phospho-vanillin assay (Osteikoetxea et al. 2015; Visnovitz et al. 2019) can be used to estimate the lipid content, but as discussed below, this method has several weaknesses.

The sulfo-phospho-vanillin assay was initially developed in 1937 to estimate the lipid content of serum, whereas the reaction mechanism leading to the pink-coloured complex quantified at 520–530 nm was first studied by two groups in the 1970s (Knight et al. 1972; Johnson et al. 1977). Although many lipids give rise to the pink-coloured complex, this is not a good method for quantification of total lipids due to several reasons. Only unsaturated lipids (having a carbon–carbon double bond) contribute to the pink colour; saturated lipids do not react (Knight et al. 1972; Johnson et al. 1977). Also, lipids having more than one double bond such as polyunsaturated fatty acids or fatty acyl groups result in less colour production than monounsaturated lipids (Knight et al. 1972, Johnson et al. 1977). Furthermore, it has been shown that a mixture of unsaturated (e.g. oleic acid, C18:1) and saturated (e.g. stearic acid, C18:0) fatty acids resulted in a colour formation that was not proportional to the ratio of these two lipids (Knight et al. 1972). Thus, although the sulfo-phospho-vanillin assay is relatively rapid and simple and gives a nice linear standard curve with only small deviations between replicates, when, for example, oleic acid is used as the standard, this method should not be recommended to quantify the total lipid content of biological samples containing a large number of unknown lipid molecules as in the case for EV preparations. For a more extensive discussion and the challenges with this method, see Knight et al. (1972), Johnson et al. (1977) and also a new article

20013078, 2025, 3, Downloaded from https://isevjournals.onlinelibrary.wiley.com/doi/10.1002/jev2.70049 by Norwegian Institute Of Public Healt Invoice Receipt DFO, Wiley Online Library on [17/03/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

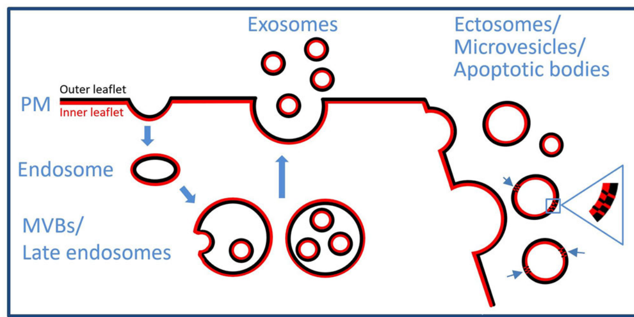

FIGURE 2 | Illustration of the asymmetric distribution of lipids in the two leaflets of membrane bilayers. In a healthy cell the outer leaflet of the PM (black) contains almost all sphingolipids and PC, whereas the inner leaflet (red) contains the other phospholipids such as PE, PS and PI. In early endosomes and in the outer membrane of MVBs (late endosomes), the 'red leaflet' is still facing cytosol, whereas in ILVs (and thus in exosomes) the red leaflet faces the lumen of these vesicles. Thus, the asymmetry in exosomes and the plasma membrane is similar at the time of secretion. We have not included in this illustration EVs excreted from other intracellular origins than MVBs as more information is needed to clarify how these EVs are secreted. Many types of EVs can be released directly from the PM (see the main text). These EVs include vesicles released from cells undergoing death processes, for example, apoptosis or ferroptosis, that is, vesicles known to have PS on their surface (only apoptotic bodies listed in the figure). The structure of membrane areas with PS on the surface is not known, but we have illustrated such EVs by mixing the red and black leaflets in small areas marked with arrows and enlarged one such area in the inset.

(Bailey et al. 2022) where this method was optimized to obtain a first estimate of the lipid content before analysing the sample with liquid chromatography coupled with mass spectrometry detection.

3 | Mass Spectrometry-Based Methods for Analyses of Lipids

The obvious method of choice for quantification and characterization of the lipid content of biological samples like EVs is mass spectrometry (MS). There are many different types of such analyses which today can be used to quantify several hundred or close to a thousand lipid species (lipids containing different fatty acyl groups) of the various lipid classes (Wenk 2010; Jung et al. 2011). Cells contain several thousand lipid species; we recommend an excellent review article by Harayama and Riezman to scientists who want to learn about the diversity and compositions of membrane lipids (Harayama and Riezman 2018). As shown in Table 1 and Figure 1, the hydrophobic chains can be bound to glycerol in the phospholipids either as a fatty acyl ester or with an ether linkage. There has been relatively little focus so far on ether lipids in biology; for a discussion of the lipid composition and the role of ether lipids common in EVs, see Skotland et al. (2019). It is a challenge to identify lipid species since there are many lipids with similar molecular masses. Thus, standardization of the MS analyses and robust quality control mechanisms are important as discussed by the lipid standard initiative (McDonald et al. 2022; Kopczynski et al. 2024).

We will not discuss these methods in detail here, but just refer to several excellent reviews describing how to perform such analyses. The use of various MS methods, sample preparation, workflow, and the use of internal standards are discussed in, for example, Wang et al. (2017, 2019), Holcapek et al. (2018), Zullig et al. (2020). The expected accuracy of quantitative lipid analyses and the need for correcting for the isotope effect due to the presence of ^{13}C is discussed in Yang and Han (2011) and the reproducibility in Jung et al. (2011). Correction for the isotope effect is important when quantifying lipid species since the presence of two ^{13}C atoms gives the same mass increase as two H atoms, that is, the difference due to a double bond. As discussed in detail elsewhere (Skotland and Sandvig 2022), lipids with one double bond (e.g., PS 18:0/18:1) are very common in biology (often making up to half of all PS species), and the isotope effect in this species may wrongly indicate the presence of a fully saturated lipid (such as PS 18:0/18:0), which normally is present in extremely low amounts and often is not detectable. If not correcting for the isotope effect the data indicate the presence of saturated species at about 10% of the amount of the species with one double bond. We have recently discussed various issues related to quantitative analyses of lipid species (Skotland et al. 2023) and the importance of such analyses in studies of EVs (Skotland et al. 2020).

Lipidomics analyses can be highly quantitative, and give reproducible results among different laboratories. In a recent study (Torta et al. 2024), aliquots of a single human blood plasma reference standard were sent to 34 laboratories in 19 countries. Ceramide lipids were then measured by different MS methods. The authors reported an intra-laboratory coefficient of variation of 3.7%–4.2% and an inter-laboratory variation of less than 14%. These results underscore the strong quantitative performance of MS-based lipid analyses and show that reference samples allow standardization between laboratories.

Using MS-based methods to quantify lipid species is important not only to obtain the amounts of each lipid, but also to obtain information about the potential origin of the EVs, their content and possible contaminations in the EV sample (Skotland et al. 2017, 2020); see Table 2. The possibility to use MS-based methods for such purposes has been greatly overlooked in most of the EV literature as discussed below. Although not being the main focus of the present article, it should be mentioned that quantitative MS-based lipid analyses of EVs have been of great value to learn about membrane structure and function in general as one can estimate the ratio of the various lipid molecules in the single lipid bilayer (Skotland et al. 2023).

The surface area of the inner and outer leaflets will not be similar for small EVs. By assuming a membrane thickness of 5 nm, EVs with a diameter of 50–100 nm will have an outer surface area, that is, 1.56–1.24 larger than the inner surface area (Skotland et al. 2017). Thus, quantifications of the various lipid classes in EVs can be used to obtain information both about the quality of the MS analyses and the purity of the EV preparations as discussed previously (Skotland et al. 2017, 2020). Comparison of the contribution of the different lipid classes to the total lipid composition of EVs and the cells they are released from can be very useful. Most data presented from such analyses demonstrate an enrichment of sphingolipids (such as sphingomyelin; SM),

20013078, 2025, 3, Downloaded from https://isevjournals.onlinelibrary.wiley.com/doi/10.1002/jev2.70049 by Norwegian Institute Of Public Healt Invoice Receipt DFO, Wiley Online Library on [17/03/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 2 | Lipid classes mentioned in this article. Note that the lipids in the exosome membrane should be expected to have the same orientation as in the plasma membrane (PM). TG, CE and cardiolipin are not present in the PM. The presence of these lipids in EV preparations may give important information about the origin of the EVs or subfractions of the EV samples analysed. TG and CE in EV samples may also originate from plasma or serum lipoproteins.

Lipid class	Present in the PM		Main intracellular localization for the lipids not present in the PM
	Yes/No	**Main leaflet**	
Sphingolipids	Yes	Outer	
PC	Yes	Outer	
PE	Yes	Inner	
PS	Yes	Inner	
PI	Yes	Inner	
TG	No		Lipid droplets
CE	No		Lipid droplets
Cardiolipin	No		Mitochondria

cholesterol, and PS (often by a factor of 2–3), whereas PC and PI are enriched in cells (Skotland et al. 2017, 2020).

Articles in which the authors compared the lipid composition of EVs and the cells of origin, often contain data showing a major enrichment for triacylglycerol (TG; in biological literature often abbreviated TAG) and/or cholesterol esters (CE). These enrichments may even be a factor of 20 times or higher, which is much higher than reported for sphingolipids, cholesterol, and PS. As TG and CE are not part of the membrane bilayer of EVs, the enrichment of these lipids provides information about the origin of the EVs or reveals the presence of possible contaminants that have been co-isolated with the EVs. TG and CE in EV preparations may originate from lipid droplets and could be excreted via secretory autophagy or be present as part of lipoproteins copurified with EVs (Skotland et al. 2017, 2020). Lipid droplets are surrounded by a lipid monolayer that is rich in PC and contains very little SM, PS and cholesterol (Wolk and Fedorova 2024). EV preparations containing a large amount of lipid droplets may thus show different enrichments from cells to EVs of these lipids compared to that discussed above. Work is ongoing in several laboratories to develop methods to remove lipoproteins from EV samples (Chou et al. 2024). If all lipoproteins can be removed from such samples, the presence of TG or CE must originate from lipid droplets.

Another lipid reported to be present in EV samples and which is not expected to be present in EV membranes is cardiolipin, which normally is present only in the inner membrane of mitochondria (Paradies et al. 2019). The presence of cardiolipin in EV samples is most likely due to parts of mitochondria being sequestered by autophagy, followed by excretion from cells by secretory autophagy. Autophagy of mitochondria, called mitophagy, is a mechanism that has gained much attention during the last years (Lu et al. 2023). Quantification of cardiolipin, TG and CE in EV preparations thus provide valuable information about the formation of the EVs, as well as processes in the cells of origin. Clearly, the presence of these lipids in EV preparations should be discussed, but this is seldom done. This discussion demonstrates that TG, CE and cardiolipin should be included in lipidomic analyses of EVs.

A change in the lipid composition of EVs may reveal an effect on cellular physiology caused by specific treatments of the cells or a change in metabolic activity. An example is that incubation of cells with the ether-lipid precursor hexadecylglycerol resulted in an increased level of ether-linked lipids in the cells and an increased release of EVs containing more ether-linked lipids as well as a changed protein composition (Phuyal et al. 2015). It is reasonable to believe that changes in lipid composition can be used as biomarkers and that they may affect EV stability and the ability of EVs to deliver drugs (Ghadami and Dellinger 2023).

Although MS-based analyses are a key to quantify almost all lipids discussed in this article it should be mentioned that free cholesterol can be quantified using commercially available kits. Moreover, by dividing the sample in two and treating one of the samples with cholesterol esterase to remove the fatty acyl group, both the amount of free cholesterol and the sum of cholesterol and CE can be quantified.

4 | Need for Improvement of MS-Based Lipid Analyses

Despite the many excellent articles mentioned above regarding how to perform MS-based lipid analyses, published data often contain lipid species that are highly unlikely to be present in the samples (Wood and Cebak 2018; Kofeler et al. 2021). The main reason for such mistakes is probably that there are many lipid species with similar molecular masses and that investigators compare the MS signals (m/z values) with data found in software tools without performing the necessary quality control of their data, and without having sufficient knowledge about which lipid species that are likely to be present in the samples. To improve the quality of MS-based lipid analyses a group of experts in the field established the Lipidomic Standard Initiative. This group has published, for example, recommendations for good practice in MS-based lipid analyses (Kofeler et al. 2021) and introduced a lipidomic minimal reporting checklist (McDonald et al. 2022; Kopczynski et al. 2024). We advise people planning to perform MS-based lipid analyses without being experienced with such

20013078, 2025, 3, Downloaded from https://isevjournals.onlinelibrary.wiley.com/doi/10.1002/jev2.70049 by Norwegian Institute Of Public Healt Invoice Receipt DFO, Wiley Online Library on [17/03/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

analyses to read the guidelines and recommendations found at their webpage (lipidomicstandards.org).

We have in several articles critically reviewed lipid data published for EVs (Skotland et al. 2017, 2020; Skotland and Sandvig 2022), and we refer to these articles regarding discussions of the many unlikely or erroneous data published for lipids in EVs. Furthermore, we recently published a short article together with scientists from the consortium of the lipidomic standard initiative where we describe pitfalls in MS-based lipid analyses and present a brief guide for biologists about how to avoid these pitfalls (Skotland et al. 2024). The article also includes simple guidelines for assessing the likelihood of lipid species to be present in mammalian samples. The hope is that this initiative will help scientists submitting lipid data as well as reviewers, editors and readers evaluating these data, to reduce the reporting of erroneous lipid species.

5 | Summary

The importance of performing quantitative lipidomics of EVs and cells in an optimal way has been discussed. Scientists planning to perform MS-based lipid analyses without being experienced in the field should read the recommendations published on the homepages of the Lipidomic Standard Initiative and collaborate with experts in the field. In addition, we have discussed that enrichment from cells to EVs of TG and/or CE, which are not part of the membrane bilayer, provides useful information regarding the purity and origin of the EV preparation and that the presence of cardiolipin may indicate mitophagy and release of mitochondrial membrane by secretory autophagy. Finally, it should be checked that the sum of the quantified lipid classes in EVs agrees with what can be expected in the two leaflets of a physiological membrane.

Author Contributions

Tore Skotland: Conceptualization (lead), writing–original draft (lead), writing–review and editing (equal). **Kim Ekroos**: Writing–review and editing (equal). **Alicia Llorente**: Writing–review and editing (equal). **Kirsten Sandvig**: Writing–original draft (lead), writing–review and editing (equal).

Acknowledgements

We thank our collaborators who during the years have contributed to discussions and studies of lipids.

Conflicts of Interest

Kim Ekroos is the owner of Lipidomics Consulting Ltd. The other authors declare no competing interest.

Data Availability Statement

The authors have nothing to report.

References

Antimisiaris, S. G., S. Mourtas, and A. Marazioti. 2018. "Exosomes and Exosome-Inspired Vesicles for Targeted Drug Delivery." *Pharmaceutics* 10: 218.

Ariotti, N., Y. Wu, S. Okano, et al. 2020. "An Inverted CAV1 (Caveolin 1) Topology Defines Novel Autophagy-Dependent Exosome Secretion From Prostate Cancer Cells." *Autophagy* 17: 1–17.

Atkin-Smith, G. K., S. Paone, D. J. Zanker, et al. 2017. "Isolation of Cell Type-Specific Apoptotic Bodies by Fluorescence-Activated Cell Sorting." *Scientific Reports* 7: 39846.

Bailey, L. S., D. V. Prajapati, and K. B. Basso. 2022. "Optimization of the Sulfo-Phospho-Vanillin Assay for Total Lipid Normalization in Untargeted Quantitative Lipidomic LC-MS/MS Applications." *Analytical Chemistry* 94: 17810–17818.

Balla, T. 2013. "Phosphoinositides: Tiny Lipids With Giant Impact on Cell Regulation." *Physiological Reviews* 93: 1019–1137.

Chou, C. Y., P. C. Chiang, C. C. Li, et al. 2024. "Improving the Purity of Extracellular Vesicles by Removal of Lipoproteins From Size Exclusion Chromatography- and Ultracentrifugation-Processed Samples Using Glycosaminoglycan-Functionalized Magnetic Beads." *ACS Applied Materials and Interfaces* 16: 44386–44398.

Ghadami, S., and K. Dellinger. 2023. "The Lipid Composition of Extracellular Vesicles: Applications in Diagnostics and Therapeutic Delivery." *Frontiers in Molecular Biosciences* 10: 1198044.

Harayama, T., and H. Riezman. 2018. "Understanding the Diversity of Membrane Lipid Composition." *Nature Reviews Molecular Cell Biology* 19: 281–296.

Hessvik, N. P., A. Overbye, A. Brech, et al. 2016. "PIKfyve Inhibition Increases Exosome Release and Induces Secretory Autophagy." *Cellular and Molecular Life Sciences* 73: 4717–4737.

Holcapek, M., G. Liebisch, and K. Ekroos. 2018. "Lipidomic Analysis." *Analytical Chemistry* 90: 4249–4257.

Johnson, K. R., G. Ellis, and C. Toothill. 1977. "The Sulfophosphovanillin Reaction for Serum Lipids: A Reappraisal." *Clinical Chemistry* 23: 1669–1678.

Jung, H. R., T. Sylvanne, K. M. Koistinen, K. Tarasov, D. Kauhanen, and K. Ekroos. 2011. "High Throughput Quantitative Molecular Lipidomics." *Biochimica Et Biophysica Acta* 1811: 925–934.

Khaldoun, S. A., M. A. Emond-Boisjoly, D. Chateau, et al. 2014. "Autophagosomes Contribute to Intracellular Lipid Distribution in Enterocytes." *Molecular Biology of the Cell* 25: 118–132.

Knight, J. A., S. Anderson, and J. M. Rawle. 1972. "Chemical Basis of the Sulfo-Phospho-Vanillin Reaction for Estimating Total Serum Lipids." *Clinical Chemistry* 18: 199–202.

Kofeler, H. C., R. Ahrends, E. S. Baker, et al. 2021. "Recommendations for Good Practice in MS-Based Lipidomics." *Journal of Lipid Research* 62: 100138.

Kofeler, H. C., T. O. Eichmann, R. Ahrends, et al. 2021. "Quality Control Requirements for the Correct Annotation of Lipidomics Data." *Nature Communications* 12: 4771.

Kopczynski, D., C. S. Ejsing, J. G. McDonald, et al. 2024. "The Lipidomics Reporting Checklist a Framework for Transparency of Lipidomic Experiments and Repurposing Resource Data." *Journal of Lipid Research* 65: 100621.

Lötvall, J., A. F. Hill, F. Hochberg, et al. 2014. "Minimal Experimental Requirements for Definition of Extracellular Vesicles and Their Functions: A Position Statement From the International Society for Extracellular Vesicles." *Journal of Extracellular Vesicles* 3: 26913.

Lu, Y., Z. Li, S. Zhang, T. Zhang, Y. Liu, and L. Zhang. 2023. "Cellular Mitophagy: Mechanism, Roles in Diseases and Small Molecule Pharmacological Regulation." *Theranostics* 13: 736–766.

McDonald, J. G., C. S. Ejsing, D. Kopczynski, et al. 2022. "Introducing the Lipidomics Minimal Reporting Checklist." *Nature Metabolism* 4: 1086–1088.

20013078, 2025, 3, Downloaded from https://isevjournals.onlinelibrary.wiley.com/doi/10.1002/jev2.70049 by Norwegian Institute Of Public Healt Invoice Receipt DFO, Wiley Online Library on [17/03/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Merrill A. H. Jr. 2011. "Sphingolipid and Glycosphingolipid Metabolic Pathways in the Era of Sphingolipidomics." *Chemical Reviews Journal* 111: 6387–6422.

Mihály, J., R. Deák, I. C. Szigyártó, A. Bóta, T. Beke-Somfai, and Z. Varga. 2017. "Characterization of Extracellular Vesicles by IR Spectroscopy: Fast and Simple Classification Based on Amide and CH Stretching Vibrations." *Biochimica Biophysica Acta Biomembranes* 1859: 459–466.

Nagata, S. 2018. "Apoptosis and Clearance of Apoptotic Cells." *Annual Review of Immunology* 36: 489–517.

Osteikoetxea, X., A. Balogh, K. Szabo-Taylor, et al. 2015. "Improved Characterization of EV Preparations Based on Protein to Lipid Ratio and Lipid Properties." *PLoS ONE* 10: e0121184.

Paradies, G., V. Paradies, F. M. Ruggiero, and G. Petrosillo. 2019. "Role of Cardiolipin in Mitochondrial Function and Dynamics in Health and Disease: Molecular and Pharmacological Aspects." *Cells* 8, no. 7: 728.

Phuyal, S., T. Skotland, N. P. Hessvik, et al. 2015. "The Ether Lipid Precursor Hexadecylglycerol Stimulates the Release and Changes the Composition of Exosomes Derived From PC-3 Cells." *Journal of Biological Chemistry* 290: 4225–4237.

Sezgin, E., T. Sadowski, and K. Simons. 2014. "Measuring Lipid Packing of Model and Cellular Membranes With Environment Sensitive Probes." *Langmuir* 30: 8160–8166.

Skotland, T., K. Sandvig, and A. Llorente. 2017. "Lipids in Exosomes: Current Knowledge and the Way Forward." *Progress in Lipid Research* 66: 30–41.

Skotland, T., N. P. Hessvik, K. Sandvig, and A. Llorente. 2019. "Exosomal Lipid Composition and the Role of Ether Lipids and Phosphoinositides in Exosome Biology." *Journal of Lipid Research* 60: 9–18.

Skotland, T., K. Sagini, K. Sandvig, and A. Llorente. 2020. "An Emerging Focus on Lipids in Extracellular Vesicles." *Advanced Drug Delivery Reviews* 159: 308–321.

Skotland, T., and K. Sandvig. 2022. "Need for More Focus on Lipid Species in Studies of Biological and Model Membranes." *Progress in Lipid Research* 86: 101160.

Skotland, T., A. Llorente, and K. Sandvig. 2023. "Lipids in Extracellular Vesicles: What Can be Learnt About Membrane Structure and Function? " *Cold Spring Harbor Perspectives in Biology* 15: a041415.

Skotland, T., K. Ekroos, J. McDonald, R. Ahrends, G. Liebisch, and K. Sandvig. 2024. "Pitfalls in Lipid Mass Spectrometry of Mammalian Samples—A Brief Guide for Biologists." *Nature Reviews Molecular Cell Biology* 25: 759–760.

Thery, C., K. W. Witwer, E. Aikawa, et al. 2018. "Minimal Information for Studies of Extracellular Vesicles 2018 (MISEV2018): A Position Statement of the International Society for Extracellular Vesicles and Update of the MISEV2014 Guidelines." *Journal of Extracellular Vesicles* 7: 1535750.

Torta, F., N. Hoffmann, B. Burla, et al. 2024. "Concordant Inter-Laboratory Derived Concentrations of Ceramides in Human Plasma Reference Materials via Authentic Standards." *Nature Communications* 15: 8562.

van Meer, G., D. R. Voelker, and G. W. Feigenson. 2008. "Membrane Lipids: Where They Are and How They Behave." *Nature Reviews Molecular Cell Biology* 9: 112–124.

van Niel, G., D. R. F. Carter, A. Clayton, D. W. Lambert, G. Raposo, and P. Vader. 2022. "Challenges and Directions in Studying Cell-Cell Communication by Extracellular Vesicles." *Nature Reviews Molecular Cell Biology* 23: 369–382.

Visnovitz, T., X. Osteikoetxea, B. W. Sodar, et al. 2019. "An Improved 96 Well Plate Format Lipid Quantification Assay for Standardisation of Experiments With Extracellular Vesicles." *Journal of Extracellular Vesicles* 8: 1565263.

Wang, J., C. Wang, and X. Han. 2019. "Tutorial on Lipidomics." *Analytica Chimica Acta* 1061: 28–41.

Wang, M., C. Wang, and X. Han. 2017. "Selection of Internal Standards for Accurate Quantification of Complex Lipid Species in Biological Extracts by Electrospray Ionization Mass Spectrometry—What, How and Why? " *Mass Spectrometry Reviews* 36: 693–714.

Welsh, J. A., D. C. I. Goberdhan, L. O'Driscoll, et al. 2024. "Minimal Information for Studies of Extracellular Vesicles (MISEV2023): From Basic to Advanced Approaches." *Journal of Extracellular Vesicles* 13: e12404.

Wenk, M. R. 2010. "Lipidomics: New Tools and Applications." *Cell* 143: 888–895.

Wolk, M., and M. Fedorova. 2024. "The Lipid Droplet Lipidome." *Febs Letters* 598: 1215–1225.

Wood, P. L., and J. E. Cebak. 2018. "Lipidomics Biomarker Studies: Errors, Limitations, and the Future." *Biochemical and Biophysical Research Communications* 504: 569–575.

Yang, K., and X. Han. 2011. "Accurate Quantification of Lipid Species by Electrospray Ionization Mass Spectrometry—Meet a Key Challenge in Lipidomics." *Metabolites* 1: 21–40.

Zargarian, S., I. Shlomovitz, Z. Erlich, et al. 2017. "Phosphatidylserine Externalization, "Necroptotic Bodies" Release, and Phagocytosis During Necroptosis." *Plos Biology* 15: e2002711.

Zhang, H., D. Freitas, H. S. Kim, et al. 2018. "Identification of Distinct Nanoparticles and Subsets of Extracellular Vesicles by Asymmetric Flow Field-Flow Fractionation." *Nature Cell Biology* 20: 332–343.

Zullig, T., M. Trotzmuller, and H. C. Kofeler. 2020. "Lipidomics From Sample Preparation to Data Analysis: A Primer." *Analytical and Bioanalytical Chemistry* 412: 2191–2209.

20013078, 2025, 3, Downloaded from https://isevjournals.onlinelibrary.wiley.com/doi/10.1002/jev2.70049 by Norwegian Institute Of Public Healt Invoice Receipt DFO, Wiley Online Library on [17/03/2025]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License