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In Vitro Liquid Extraction Surface Analysis Mass Spectrometry (ivLESA-MS) for Direct Metabolic Analysis of Adherent Cells in Culture

Sankha S. Basu[†], **Elizabeth C. Randall**[‡], **Michael S. Regan**[§], **Begoña G.C. Lopez**[§], **Amanda R. Clark**[§], **Nicholas D. Schmitt**[‖,⊥], **Jeffrey N. Agar**[‖,⊥,#], **Deborah A. Dillon**[†], and **Nathalie Y. R. Agar**[*,‡,§,††]

[†]Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115, United States

[‡]Department of Radiology, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115, United States

[§]Department of Neurosurgery, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115, United States

[‖]Department of Chemistry and Chemical Biology, Northeastern University, Boston, Massachusetts 02115, United States

[⊥]Barnett Institute of Chemical and Biological Analysis, Northeastern University, Boston, Massachusetts 02115, United States

[#]Department of Pharmaceutical Sciences, Northeastern University, Boston, Massachusetts 02115, United States

[††]Department of Cancer Biology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115, United States

Abstract

Conventional metabolomic methods include extensive sample preparation steps and long analytical run times, increasing the likelihood of processing artifacts and limiting high throughput applications. We present here in vitro liquid extraction surface analysis mass spectrometry (ivLESA-MS), a variation on LESA-MS, performed directly on adherent cells grown in 96-well cell culture plates. To accomplish this, culture medium was aspirated immediately prior to analysis, and metabolites were extracted using LESA from the cell monolayer surface, followed by nano-electrospray ionization and MS analysis in negative ion mode. We applied this platform to characterize and compare lipidomic profiles of multiple breast cancer cell lines growing in culture

[*]**Corresponding Author:** Mailing Address: Nathalie Y.R. Agar, Ph.D., Hale Building for Transformative Medicine, 60 Fenwood Road, Suite 8016-J, Boston, Massachusetts 02115, United States; Tel: 617.525.7374; Fax: 617.264.6316; Nathalie_Agar@dfci.harvard.edu.

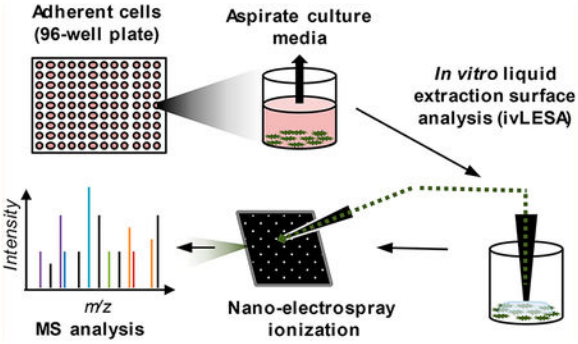
Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.anal-chem.8b00530. Photos of ivLESA interface; mass spectra demonstrating method reproducibility; methods and data for lipid extraction; ESI-FTICR-MS confirmation of metabolites of interest (PDF)

The authors declare no competing financial interest.

(MCF-7, ZR-75–1, MDA-MB-453, and MDA-MB-231) and revealed distinct and reproducible lipidomic signatures between the cell lines. Additionally, we demonstrated time-dependent processing artifacts, underscoring the importance of immediate analysis. ivLESA-MS represents a rapid in vitro metabolomic method, which precludes the need for quenching, cell harvesting, sample preparation, and chromatography, significantly shortening preparation and analysis time while minimizing processing artifacts. This method could be further adapted to test drugs in vitro in a high throughput manner.

Graphical Abstract



Altered cellular metabolism is a well-known hallmark of cancer[1] and a subject area that has received renewed interest as a target for cancer therapy.[2] Accordingly, robust in vitro platforms to quickly and reproducibly assess cellular metabolism are strongly desired. Unfortunately, conventional metabolic analysis of adherent cells can be both time and labor intensive and often involves extensive sample preparation steps, chemical derivatization, as well as liquid or gas chromatography prior to mass spectral (MS) analysis. Although these steps improve the sensitivity and specificity for many analytes, they can hamper applications that require high throughput or rapid analysis. Additionally, it has been shown that processing steps such as washing, scraping, and trypsinization can quickly lead to metabolic changes, thereby introducing processing artifacts.[3,4]

To minimize sample preparation and accelerate analysis, a variety of ambient ionization platforms have been developed, including a number of techniques involving spray desorption and liquid microjunction-based interfaces.[5] One robust liquid microjunction-based platform is liquid extraction surface analysis (LESA),[6] in which the extraction solvent is applied directly to the sample of interest, and the extracted analytes are subsequently introduced into the mass spectrometer using nano-electrospray ionization (nanoESI). The sensitivity, automated interface, solvent flexibility, and negligible carryover makes LESA particularly well suited for translational and clinical applications.

The majority of LESA applications published to date have analyzed biological samples affixed to glass slides.[7] Although glass slides are ideal for tissue sections, they are not the optimal surface for cell culture analysis, as they would require either harvesting and transferring cells to slides or growing the cells on the slides themselves. In the former, each additional processing step increases the likelihood of introducing processing artifacts arising from metabolic perturbation or analyte instability. As far as growing cells on glass slides,

this presents scale-up and reproducibility challenges, and not all cells grow well on glass surfaces. Therefore, an optimal platform would involve direct analysis of cells in culture, with as few processing steps as possible.

Here, we present in vitro LESA-MS (ivLESA-MS), an adaptation of LESA-MS, in which direct metabolic analysis is performed on adherent cells grown on standard 96-well cell culture plates, precluding the need for cell harvesting, protracted processing steps, or chromatographic separation. The primary benefits include decreasing processing and analysis time as well as minimizing metabolic artifacts that can be generated during sample processing.

EXPERIMENTAL SECTION

Chemicals and Materials.

Ammonium acetate, methanol, and isopropanol (HPLC grade or higher) were obtained from Fisher Scientific (Pittsburgh, PA), and chloroform was obtained from Sigma-Aldrich (St. Louis, MO). Dulbecco's minimum essential media (DMEM) with high glucose (4.5 g/L), Roswell Park Memorial Institute (RPMI) 1640 media, fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Thermo Fisher Scientific (Carlsbad, CA).

Cell Culture.

We performed our cell culture experiments using the following breast cancer cell lines: MCF-7, ZR-75–1, MDA-MB-453, and MDA-MB-231 (kindly provided to us by the laboratory of Dr. Joan Brugge, Harvard Medical School, Boston, MA). Characteristics of these cells are described in Table 1. MCF-7, MDA-MB-231, and MDA-MB-453 cells were grown in DMEM media, supplemented with 10% heat inactivated FBS, 100 units per/mL penicillin, and 100 μg/mL streptomycin. ZR-75–1 cells were maintained in RPMI media, containing the same additives. Cells were maintained and subcultured in T-25 flasks and plated in standard 96-well flat bottom polystyrene cell culture treated plates (Corning Costar, #3599). Metabolic analysis was performed when cells were approximately 90% confluent.

ivLESA.

To perform ivLESA, we used a TriVersa NanoMate (Advion, Ithaca, NY) equipped with a nanoESI source. Analyte extraction was accomplished using a solvent system containing 15:35:50 (v/v/v) chloroform/methanol/isopropanol with 7.5 mM ammonium acetate. To allow direct analysis on the adherent cell monolayer surface, we elevated the plate using three spacers, each made up of three stacked standard glass slides and measuring 3 mm in total height. Immediately prior to analysis, culture medium was aspirated from the wells being analyzed. LESA sample parameters were as follows: solvent volume (4 μL), dispense volume (1.7 μL), postdispense delay (1.0 s), aspiration volume (2.0 μL), repeat mix (×2), postaspiration delay (1.0 s), dispensation height (−9.4 mm), aspiration height (−9.6 mm). Delivery parameters included gas pressure (0.3 psi), voltage (1.4 kV), and negative electrospray mode.

MS Analysis.

For MS analysis, the NanoMate was coupled to an ion trap mass spectrometer (amaZon speed, Bruker Daltonics, Billerica, MA) using a nanoESI source. MS acquisition was performed using trapControl software with the following parameters: negative ion polarity, end plate offset (200 V), target ions (70 000), accumulation time (50 ms), mass scan range (m/z 100−1100), and enhanced mass resolution mode. Total injection time was 1 min per sample. Spectra were analyzed using Data Analysis software (Bruker Daltonics, version 4.2), and individual ions were quantified and represented as a percentage of the total ion count (TIC).

RESULTS AND DISCUSSION

Adapting LESA to ivLESA.

To adapt LESA to ivLESA, we elected to use 96-well cell culture plates, due in part to their capacity for high throughput applications. Additionally, the LESA TriVersa NanoMate system is already adapted for direct infusion mass spectrometry (DIMS), in which liquid samples are analyzed from 96-well round-bottom plates.[8] Since the 96-well round-bottom plates have the same physical dimensions as the 96-well flat bottom cell culture plates, the only modification needed was to raise the culture plates to facilitate solvent dispensation and aspiration on the cell monolayer surface (for visualization of plate setup, see Supplemental Figure 1 in Supporting Information). Although this was effective for 96-well plates, other plate formats such as 6-, 12-, and 24-well plates were too tall to allow stage movement, so additional modifications to either the plates or the tray holder would be required to accommodate these plates. Prior to ivLESA-MS analysis, cells were grown to near confluency, and the culture medium was manually aspirated. To extract fatty acids and lipids, 1.7 μL of organic solvent was dispensed directly onto the cell monolayer surface, and this was repeated a second time for more complete extraction. The solvent was then aspirated and injected into the MS using nanoESI. By using a lower aspiration height (−9.6 mm) compared to the dispensation height (−9.4 mm), we achieved a more reliable signal with fewer missed samples resulting from instability of the liquid microjunction and associated solvent spread. This is of particular importance for solvents that have low viscosity and surface tension, such as those used in this study. A schematic of the ivLESA is provided in Figure 1.

Optimization and Analytical Characteristics of ivLESA.

One of the primary difficulties of conventional metabolic analyses is the generation of processing artifacts, often introduced by improper quenching, washing, scraping, or trypsinizing.[4] By using ivLESA, these steps were essentially eliminated since analyte extraction was performed directly on the adherent cells. Nevertheless, in each cell line tested, we found temporal changes in the relative signal intensity of certain metabolites if there was a significant delay between media aspiration and ivLESA-MS analysis. For example, within 15–30 min after aspiration, we reproducibly observed a 7–30 fold increase in the relative signal intensity of m/z 281.2 (oleic acid), with a 50–90% decrease in the signal intensity for 885.5 (PI 38:4) among the cell lines (Figure 2). However, we did not see similar changes at early time points. It should be noted that although similar trends in time-

dependent changes were seen in all tested cell lines, some of the relative signal intensity changes could be due to ion suppression effects, which are not uncommon in surface sampling ionization techniques. Nonetheless, subsequent ivLESA-MS analyses were performed immediately after media aspiration. Moreover, future in vitro and ex vivo analyses should consider and account for such metabolic changes that may occur in the analysis of fresh specimens.

In addition to minimizing metabolic changes during processing, reducing analytical time was another critical goal of this approach. As such, we wanted to determine the minimum run time needed for an individual well. The total preanalytical LESA interval (including tip and solvent acquisition, as well as solvent dispensation, aspiration, and nanoESI introduction of extract) was approximately 45 s per sample. The aspirated solvent was subsequently injected and analyzed by MS for 1 min, providing a combined preparation and analysis time of under 2 min. Therefore, using this approach, a full 96-well plate could be analyzed in just over 3 h. For comparison, a more traditional lipidomics approach using liquid chromatography/tandem mass spectrometry (LC-MS/MS) analysis of adherent cells would require metabolic quenching, cell harvesting, centrifugation, lysis, solid-phase or liquid −liquid extraction, solvent drying, and resuspension prior to LC-MS/MS analysis, the latter of which alone could take 30 min per sample. Therefore, using conventional methods, a 96-well plate could take more than 24 h to fully analyze, not to mention the significant labor, supply, and reagent costs.

Finally, to assess the precision of ivLESA-MS, we performed analysis on multiple wells (n = 4) of MDA-MD-231 breast cancer cells and demonstrated highly reproducible lipidomic signatures (see Supplemental Figure 2 in Supporting Information for full spectra). Quantitatively, the relative signal intensity, as a percent of TIC, for each of the 10 most abundant ions demonstrated a CV less than 20%, with the majority under 10% (Table 2). Moreover, the fatty acid and phospholipid profiles were similar to those we have seen in specimens analyzed in the operating room.[9−11] Taken together, ivLESAMS provides a rapid, sensitive, and precise platform to characterize metabolites extracted from adherent cells in culture, minimizing artifacts and reducing processing and analytical time.

Lipidomic Differences between Breast Cancer Cell Lines.

Compared to the extensive literature describing genomic and proteomic differences observed between different types of breast cancers and breast cancer cell lines, there are considerably fewer delineating metabolomic differences, and specifically, lipidomic differences between these cells.[12] As a proof of concept, we applied ivLESA-MS to characterize lipidomic differences between four commonly studied breast cancer cell lines, namely, MCF-7,[13] ZR-75–1,[14] MDA-MB-453,[15] and MDA-MB-231,[16] which include a range of breast cancer subtypes, from the less aggressive and more targetable MCF-7 cells, which express both an estrogen receptor (ER) and progesterone receptor (PR), to MDA-MB-231, a more aggressive triple negative breast cancer line[17,18] (Table 1).

Although we anticipated minor lipidomic differences between cell lines, we found the cell lines to have significantly different lipid signatures (Figure 3). Most notably, while the triple negative MDA-MB-231 cells demonstrated similar or lower relative signal intensities for

more common glycerophospholipid ions with *m*/*z* values of 742.5 (PE 36:2), 766.5 (PE 38:4), and 885.5 (PI 38:4), these cells exhibited multiple peaks (including 572.5, 682.5, and 684.5 *m*/*z*) at much higher relative signal intensities than other cell lines, as demonstrated in Figure 4. Further characterization of these three ions using Fourier transform ion cyclotron resonance high resolution mass spectrometry (FTICR-HRMS) identified these molecules as the chloride adducts $[M + Cl]^-$ of three different ceramide species, most likely Cer(d18:1/16:0), Cer(d18:1/24:1), and Cer(d18:1/24:0), respectively (see Supporting Information for details). The corresponding $[M - H]^-$ adducts for these molecules (536.5, 646.5, and 648.5 *m*/*z*) were also seen in much higher relative abundance in the MDA-MB-231 cells, albeit at lower signal intensities than their corresponding chloride adducts. The predominance of chloride ceramide adducts over deprotonated ions can be attributed to the presence of chloroform in the extraction solvent and has been previously reported in the literature.[19,20] The finding of elevated ceramides is significant, as ceramides not only represent a major structural lipid class but also have been associated with lipid rafts,[21] increased during apoptosis,[22,23] elevated in several cancers including breast cancer,[24,25] and play a central role in cell signaling.[26] Therefore, by using ivLESA-MS, we demonstrate a distinguishable metabolic feature in these cells, which could aid in tumor classification as well as targeted therapy.[27,28]

Limitations and Areas for Improvement.

Since this report was intended to provide a proof-of-principle for ivLESA-MS, there are predictably areas for improvement. For one, improved metabolite identification could be accomplished by connecting the ivLESA to a high resolution mass spectrometer, as has been described in the literature for LESA,[29] though bearing in mind that fragmentation and/or chromatography would still be needed to differentiate isobaric compounds. Additionally, the method presented here involved manual aspiration of the culture media prior to analysis. An instrument that could first aspirate the media and then perform LESA would be ideal. To our knowledge, such an interface is not currently commercially available. Finally, there are likely metabolic changes that occurred due to changes in the media temperature, as these cells are normally maintained at 37 °C. Therefore, to mitigate temperature-associated metabolic artifacts, a heated platform would be ideal, though we did not have this feature on our system.

CONCLUSIONS

We present here ivLESA-MS, a novel application to perform direct metabolic analysis on adherent cells in cell culture plates. By performing analysis in 96-well cell culture plates, we provide a platform that could be used for a wide variety of applications, including testing of single or combination drug therapies, functional metabolic analysis using ^{13}C-isotopic tracers, and potentially ex vivo metabolic analysis on primary cells. Additionally, we found distinct and reproducible lipid signatures among commonly studied breast cancer cell lines, which has both diagnostic and therapeutic implications. Future directions include optimizing the platform for monitoring organic acids, carbohydrates, proteins, and other endogenous molecules.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

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REFERENCES

(1). Hanahan D; Weinberg RA Cell 2011, 144 (5), 646–674. [PubMed: 21376230]

(2). Vander Heiden MG Nat. Rev. Drug Discovery 2011, 10 (9), 671–684. [PubMed: 21878982]

(3). de Koning W; van Dam K Anal. Biochem 1992, 204 (1), 118–123. [PubMed: 1514678]

(4). Teng Q; Huang W; Collette TW; Ekman DR; Tan C Metabolomics 2009, 5 (2), 199–208.

(5). Venter AR; Douglass KA; Shelley JT; Hasman G; Honarvar E Anal. Chem 2014, 86, 233–249. [PubMed: 24308499]

(6). Eikel D; Henion JD In Mass Spectrometry for Drug Discovery and Drug Development; Korfmacher WA, Ed.; John Wiley & Sons, Inc.: Hoboken, NJ, U.S.A., 2013; pp 221–238.

(7). Ellis SR; Brown SH; in het Panhuis M; Blanksby SJ; Mitchell TW Prog. Lipid Res 2013, 52 (4), 329–353. [PubMed: 23623802]

(8). Dethy JM; Ackermann BL; Delatour C; Henion JD; Schultz GA Anal. Chem 2003, 75 (4), 805–811. [PubMed: 12622370]

(9). Calligaris D; Caragacianu D; Liu X; Norton I; Thompson CJ; Richardson a. L.; Golshan M; Easterling ML; Santagata S; Dillon D. a.; Jolesz F. a.; Agar NYR Proc. Natl. Acad. Sci. U. S. A 2014, 111 (42), 15184–15189. [PubMed: 25246570]

(10). Eberlin LS; Norton I; Orringer D; Dunn IF; Liu X; Ide JL; Jarmusch AK; Ligon KL; Jolesz FA; Golby AJ; Santagata S; Agar NYR; Cooks RG Proc. Natl. Acad. Sci. U. S. A 2013, 110(5), 1611–1616. [PubMed: 23300285]

(11). Santagata S; Eberlin LS; Norton I; Calligaris D; Feldman DR; Ide JL; Liu X; Wiley JS; Vestal ML; Ramkissoon SH; Orringer DA; Gill KK; Dunn IF; Dias-Santagata D; Ligon KL; Jolesz FA; Golby AJ; Cooks RG; Agar NY R. Proc. Natl. Acad. Sci. U. S. A 2014, 111 (30), 11121–11126.

(12). Wenk MR Nat. Rev. Drug Discovery 2005, 4 (7), 594–610. [PubMed: 16052242]

(13). Levenson A; Jordan V Cancer Res 1997, 57 (15), 3071–3078. [PubMed: 9242427]

(14). Engel LW; Young NA; Tralka TS; Lippman ME; O'Brien SJ; Joyce MJ Cancer Res 1978, 38 (10), 3352–3364. [PubMed: 688225]

(15). Hall RE; Birrell SN; Tilley WD; Sutherland RL Eur. J. Cancer 1994, 30 (4), 484–490.

(16). Cailleau R; Olivé M; Cruciger QV In Vitro 1978, 14 (11), 911–915. [PubMed: 730202]

(17). Holliday DL; Speirs V Breast Cancer Res 2011, 13, 215. [PubMed: 21884641]

(18). Subik K; Lee JF; Baxter L; Strzepek T; Costello D; Crowley P; Xing L; Hung MC; Bonfiglio T; Hicks DG; Tang P Breast Cancer: Basic Clin. Res 2010, 4 (1), 35–41.

(19). Zehethofer N; Bermbach S; Hagner S; Garn H; Muller J; Goldmann T; Lindner B; Schwudke D; Konig P Chromatographia 2015, 78 (5−6), 403–413. [PubMed: 25750457]

(20). Zhu J; Cole RB J. Am. Soc. Mass Spectrom 2000, 11 (11), 932–941. [PubMed: 11073256]

(21). Megha; London E J. Biol. Chem 2004, 279 (11), 9997–10004. [PubMed: 14699154]

(22). Thomas RL; Matsko CM; Lotze MT; Amoscato AA J. Biol. Chem 1999, 274 (43), 30580–30588. [PubMed: 10521441]

(23). Huang W-C; Chen C-L; Lin Y-S; Lin C-F J. Lipids 2011, 2011, 1–15.

(24). Saddoughi SA; Ogretmen B Adv. Cancer Res 2013, 117, 37–58. [PubMed: 23290776]

(25). Schiffmann S; Sandner J; Birod K; Wobst I; Angioni C; Ruckhäberle E; Kaufmann M; Ackermann H; Lötsch J; Schmidt H; Geisslinger G; Grösch S Carcinogenesis 2009, 30 (5), 745–752. [PubMed: 19279183]

(26). Morad SAF; Cabot MC Nat. Rev. Cancer 2013, 13 (1), 51–65. [PubMed: 23235911]

(27). Henry B; Möller C; Dimanche-Boitrel MT; Gulbins E; Becker KA Cancer Lett 2013, 332 (2), 286–294. [PubMed: 21862212]

(28). Barth BM; Cabot MC; Kester M Anti-Cancer Agents Med. Chem 2011, 11 (9), 911–919.

(29). Randall EC; Bunch J; Cooper HJ Anal. Chem 2014, 86 (21), 10504–10510. [PubMed: 25333355]

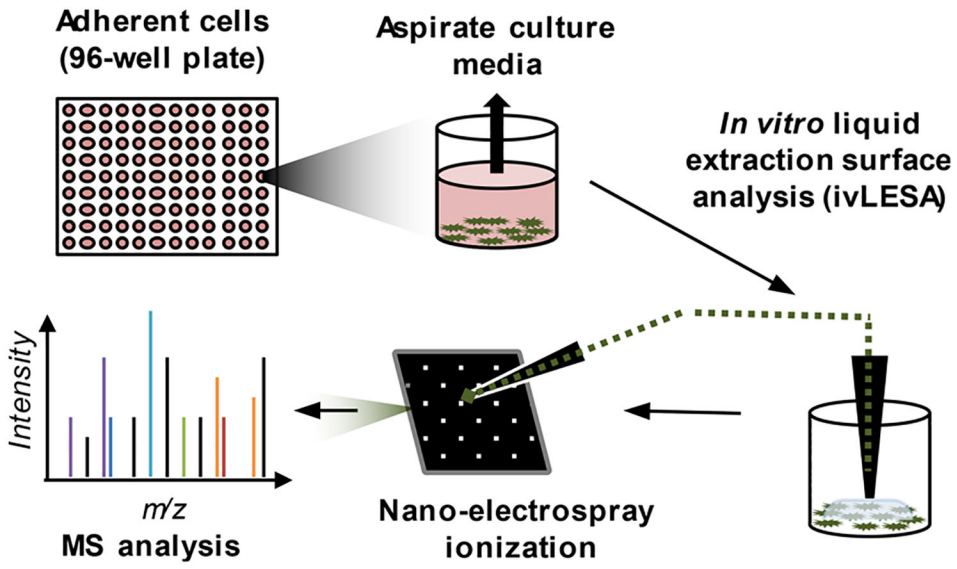

Figure 1.
Schematic of in vitro liquid extraction surface analysis (ivLESA).

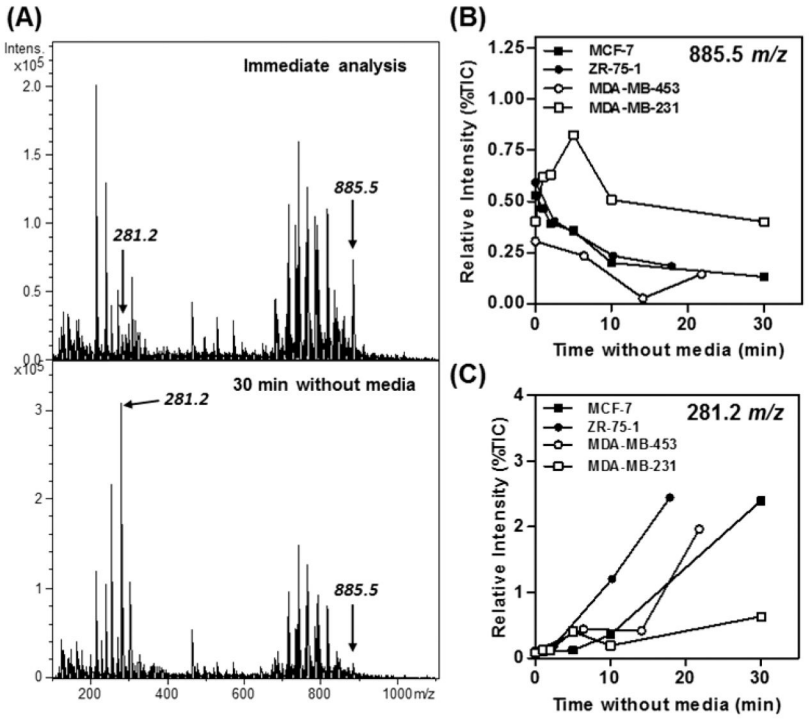

Figure 2.

Time-dependent changes in lipid profile after culture media aspiration. (A) Representative mass spectra using ivLESA-MS on MCF-7 cells analyzed either immediately after media aspiration or 30 min after culture medium was removed. Time course demonstrating changes in relative signal intensity of (B) 885.5 and (C) 281.2 *m*/*z* ions at different time intervals postaspiration on different breast cancer cell lines.

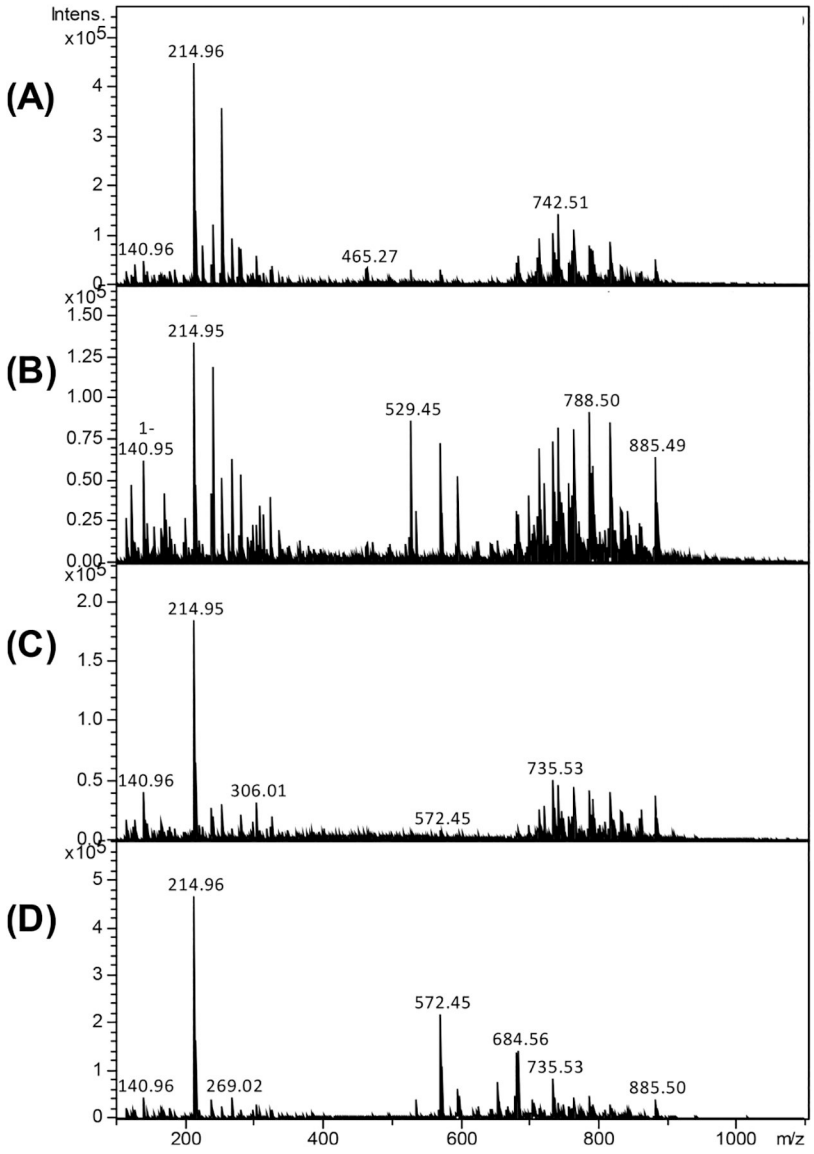

Figure 3.

ivLESA-MS spectra from four different breast cancer cell lines: (A) MCF-7, (B) ZR-75–1, (C) MDA-MB-453, (D) MDA-MB-231, demonstrating divergent lipidomic profiles.

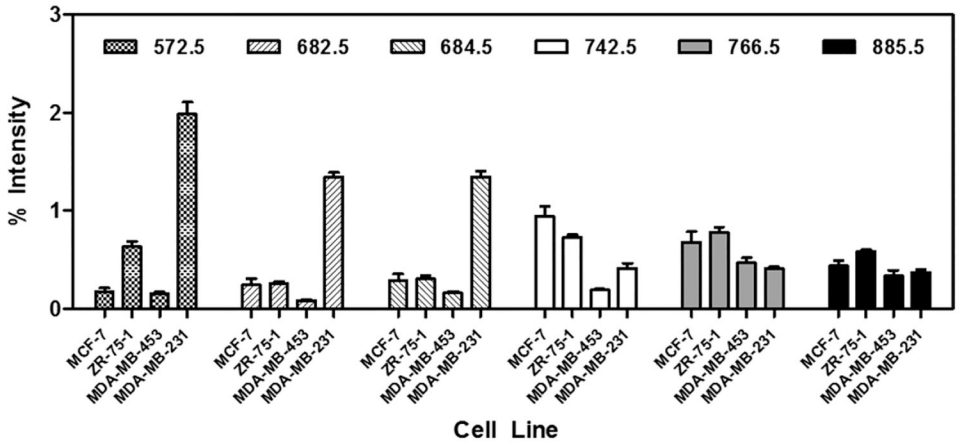

Figure 4.

Relative signal intensity of selected prominent ions in different breast cancer cell lines ($n = 4$ for each cell line). Error bars represent standard error of the mean (SEM).

Table 1.

Characteristics of Breast Cancer Cell Lines Used in This Study[a]

cell line	classification	ER	PR	Her-2	p53 Mut
MCF-7	Luminal A	+	+	−	±
ZR-75-1	Luminal B	+	−	−	−
MDA-MB-453	HER2	−	−	+	−
MDA-MB-231	Claudin-low	−	−	−	++

[a]ER: estrogen receptor; PR: progesterone receptor.

Table 2.

Precision Analysis of ivLESA-MS Method[a]

(replicate)	(1)	(2)	(3)	(4)	RSD
m/z	**signal intensity (% of TIC)**				**%CV**
215.0	4.08	3.95	4.33	4.64	**7.2**
572.5	1.82	1.78	2.03	2.31	**12.3**
684.5	1.35	1.20	1.34	1.49	**8.8**
682.5	1.27	1.32	1.31	1.48	**6.9**
574.5	1.00	0.91	1.03	1.14	**9.2**
735.5	0.58	0.73	0.78	0.67	**12.8**
656.5	0.75	0.72	0.73	0.87	**9.0**
766.5	0.46	0.40	0.41	0.38	**7.8**
885.5	0.42	0.39	0.40	0.29	**15.7**
788.5	0.48	0.45	0.47	0.39	**9.4**

[a]Top 10 MS peaks acquired by MDA-MB-231 cells grown on 96-well plates. RSD: relative standard deviation, CV: coefficient of variation, *m*/*z*: mass to charge ratio, TIC: total ion count.