Metabolic Engineering 91 (2025) 158–169

Contents lists available at ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/meteng

Modulating fatty acid metabolism and composition of CHO cells by feeding high levels of fatty acids complexed using methyl-β-cyclodextrin

Bradley Priem [a,1], Xiangchen Cai [b,1], Yu-Jun Hong [b], Karl Gilmore [a], Zijun Deng [a], Sabrina Chen [a], Harnish Mukesh Naik [a], Michael J. Betenbaugh [a], Maciek R. Antoniewicz [b,*]

[a] Department of Chemical and Biomolecular Engineering, Johns Hopkins University, Baltimore, MD, 21218, USA
[b] Department of Chemical Engineering, University of Michigan, Ann Arbor, MI, 48109, USA

ARTICLE INFO

Keywords:
Fatty acid metabolism
Mammalian cell culture
Biomass composition
^{13}C tracing
Mass spectrometry

ABSTRACT

Chinese Hamster Ovary (CHO) cells are widely used in the pharmaceutical industry to produce therapeutic proteins. Increasing the productivity of CHO cells through media development and genetic engineering is a significant industry objective. Past research demonstrated the benefits of modulating fatty acid composition of CHO cells through genetic engineering. In this study, we describe an alternative approach to modulate fatty acid composition by directly feeding high levels of fatty acids in CHO cell culture. To accomplish this, we developed and optimized a pharmaceutically relevant feeding strategy using methyl-β-cyclodextrin (MBCD) to solubilize fatty acids. To quantify fatty acid composition of CHO cells, a new GC-MS protocol was developed and validated. In fed batch cultures, we found that the degree of saturation of fatty acids in CHO cell mass, i.e. the relative abundances of saturated, monounsaturated and polyunsaturated fatty acids, can be controlled by the choice of fatty acid supplement and feeding strategy. Feeding unsaturated fatty acids such as palmitoleic acid, oleic acid, and linoleic acid had the greatest impact the fatty acid composition of CHO cells, increasing their respective abundances in cell mass by upwards of 25x, 1.5x, and 50x, respectively. ^{13}C-Tracing further revealed that the supplemented fatty acids were involved in a range of elongation, desaturation, and β-oxidation reactions to yield both common and uncommon fatty acids such as vaccenic acid and hypogeic acid. Finally, we show that CHO-K1 and CHO-GS cells take up fatty acids solubilized with MBCD at rates comparable to delivery using bovine serum albumin. Taken together, this work paves the way for new feed media formulations containing fatty acids to optimize CHO cell physiology in industrial cell cultures.

1. Introduction

In the pharmaceutical industry, Chinese Hamster Ovary (CHO) cells are responsible for producing the majority of monoclonal antibodies (mAbs), with the market expected to generate $300 billion by 2025. CHO cells are primarily used due to their ease of culture and ability to perform essential protein processing steps and post-translational modifications such as proper protein folding and glycosylation (Li et al., 2022). Increasing the productivity of CHO cells is a significant industry objective, which would reduce overall production cost by enabling more product to be generated with the same amount of cells. One major avenue to pursue this goal is media development, which involves optimizing the nutrient composition of CHO cell media to promote favorable cell metabolism, growth and product secretion (Ladiwala et al., 2024; Ritacco et al., 2018). Understanding CHO cell biology and metabolism is thus essential in properly formulating a chemically defined growth medium. Insights into glucose, amino acid and dipeptide metabolism have been used to inform media optimization strategies that have demonstrated improvements in CHO cell culture, such as increased cell density and titers, and reduced accumulation of toxic byproducts (Gonzalez et al., 2024; Ladiwala et al., 2023; Naik et al., 2023, 2024). Still, there remain media components whose impact on CHO cell metabolism and productivity have not been fully explored, including essential and non-essential fatty acids.

Fatty acids are involved in many important biological processes in mammalian cells, including in processing and secretion of proteins, generation of ATP via oxidative phosphorylation, and acting as secondary messengers in signal transduction and cell regulation pathways

* Corresponding author. Department of Chemical Engineering University of Michigan 2800 Plymouth Road, Ann Arbor, MI, 48109, USA.
E-mail address: mranton@umich.edu (M.R. Antoniewicz).
[1] Equal contribution.

https://doi.org/10.1016/j.ymben.2025.04.005
Received 6 December 2024; Received in revised form 18 March 2025; Accepted 24 April 2025
Available online 25 April 2025

(Ali et al., 2018). Additionally, fatty acids are the main components of cell membranes and changes in fatty acid composition have been shown to affect cell membrane fluidity (Maulucci et al., 2016), which in turn affects a wide range of cell membrane mediated processes such as uptake and secretion of nutrients and by-products, membrane protein localization, and protein secretion (Maulucci et al., 2016). *De novo* fatty acid biosynthesis is the process of condensing multiple acetyl-CoA (AcCoA) units into medium and long chain fatty acids. The formation of palmitic acid (C16:0) is the first step in the biosynthesis of a wide range of other long chain fatty acids, including palmitoleic acid (C16:1 n-7), stearic acid (C18:0), and oleic acid (C18:1 n-9), which are formed from palmitic acid via the actions of fatty acid elongases (ELOVL) and desaturases (SCD) (Maulucci et al., 2016). Essential fatty acids that cannot be synthesized *de novo* by CHO cells, such as linoleic acid (C18:2 n-9,12), must be supplied to CHO cells through culture medium (Whelan and Fritsche, 2013). Both essential and non-essential fatty acids can be catabolized via β-oxidation to yield AcCoA, which can then be used for ATP generation through the TCA cycle and oxidative phosphorylation, or recycled to form additional fatty acids and other biomolecules (Ma et al., 2018).

While CHO cell lipid and fatty acid compositions have been characterized before (Subbaiah et al., 2011; Szeliova et al., 2020), approaches for modulating the composition of fatty acids for improved cell culture performance remain underdeveloped. Currently, it is still unclear if or what the optimal fatty acid composition is for CHO cells to achieve desired metabolic and product secretion profiles. Promising work in this area has been described by Budge et al. that demonstrated the benefits of increasing the fraction of unsaturated fatty acids in CHO cells through the overexpression of genes associated with fatty acid and lipid metabolism such as SCD1 and SREBF1 (Budge et al., 2020). These results inspire further research into modulating fatty acid composition, particularly interventions outside of cell line engineering that could be performed without altering the genome.

Mammalian cells can incorporate small amounts of fatty acids directly from the medium (Subbaiah et al., 2011). An alternate approach to modulate CHO cell fatty acid composition would be to feed high levels of fatty acids through the medium. However, due to the low solubility of fatty acids in media, this would require solubilizing fatty acids for proper delivery to CHO cells. Bovine serum albumin (BSA) is the gold-standard for solubilizing fatty acids in aqueous solutions, although BSA is undesired in industrially regulated media formulations as it is an animal-derived product and has inherent batch-to-batch variability and can serve as a point of contamination (van der Valk et al., 2010). An alternative would be to employ a chemically defined solubilizing agent such as methyl-β-cyclodextrin (MBCD) (Zidovetzki and Levitan, 2007). In the past, MBCD has been employed to deplete and/or resupply fatty acids and cholesterol to/from mammalian cells (Ali et al., 2022; Brunaldi et al., 2010; Christian et al., 1997; Francis et al., 1999; Mahammad and Parmryd, 2015). In addition, it has been used as a vehicle for drug delivery (Stella and Rajewski, 1997). As MBCD only interacts with the plasma membrane surface, it is a useful compound to alter plasma membrane composition without entering the cell and directly disrupting metabolic activities.

In this study, we developed a pharmaceutically relevant feeding strategy to supply high levels of fatty acids to CHO cells. Specifically, we focused delivering high levels of C16 and C18 fatty acids, since these are the dominant fatty acids in CHO cells (Ali et al., 2018; Subbaiah et al., 2011). Moreover, we used ^{13}C-tracing to elucidate how the supplemented fatty acids were taken up, how biomass composition of CHO cells was affected, and how these fatty acids were further metabolized to other common and uncommon fatty acids (Gonzalez and Antoniewicz, 2017). Importantly, we show that the degree of saturation of fatty acids in CHO cell mass, i.e. the relative abundances of saturated, mono-unsaturated and poly-unsaturated fatty acids, can be controlled by the choice of fatty acid supplement and feeding strategy. Taken together, our work demonstrates that fatty acid feeding using MBCD is a viable strategy to modulate CHO cell fatty acid composition and direct fatty acid metabolism.

2. Results and discussion

2.1. Optimizing delivery of fatty acids in CHO cell culture using methyl-β-cyclodextrin

The main aim of this work was to deliver sufficiently high amounts of fatty acids in CHO cell cultures to allow us to modulate the fatty acid composition of CHO cells. Assuming CHO cells have a dry cell mass of approximately 320 pg/cell and that lipids make up about 10 % of dry cell mass (Antoniewicz, 2018), we estimated the need to deliver approximately 0.02 mg of fatty acids per million CHO cells. Thus, even for a relatively low cell density of 1 million cells/mL, this corresponds to 20 mg/L of fatty acids, which is significantly above the solubility limit for fatty acids in cell culture media. As an example, medium chain length fatty acids typically have solubility limits below 2 mg/L, and long chain fatty acids below 0.2 mg/L. Therefore, in order to deliver enough fatty acids to CHO cell cultures we needed to consider complexation strategies for optimal feeding of fatty acids.

Methyl-β-cyclodextrin (MBCD) is a cyclic oligosaccharide with a hydrophobic core that can enhance fatty acid solubility in aqueous solutions. However, MBCD can be toxic to mammalian cells due to its ability to sequester cholesterol from cell membranes (Mahammad and Parmryd, 2015; Zidovetzki and Levitan, 2007). We thus had to first evaluate the toxicity of MBCD to CHO cells. We also tested the toxicity of ethanol, since ethanol will be used in this study for preparing concentrated fatty acid stock solutions, i.e. most fatty acids are highly soluble in ethanol at concentrations above 20 g/L. Fig. 1 shows cell growth of CHO-K1 cells and CHO-GS cells in batch cultures at various levels of ethanol (up to 2 % v/v) and MBCD (up to 0.3 % w/v). For ethanol, no effect on CHO cell growth was observed up to 0.5 % ethanol (Fig. 1A). At 1 % of ethanol, we observed about 15 % reduction is growth, and at 2 % ethanol we observed significant inhibition of cell growth for both CHO cell lines. For MBCD, no effect on CHO cell growth was observed up to 0.1 % MBCD (Fig. 1B). At 0.2 % MBCD, cell growth was reduced by 20 % or more, and almost no growth was observed at 0.3 % MBCD. Based on these results, we selected 0.1 % MBCD and 0.5 % ethanol as the upper limits for the remainder of this study.

Complexation of fatty acids requires 2 to 5 molecules of MBCD per fatty acid molecule (Zidovetzki and Levitan, 2007). Thus, at the highest viable concentration of MBCD of 0.1 % w/v (or 0.76 mmol/L) we should be able to solubilize up to 150 μmol/L, or about 40 mg/L of fatty acids, which should be sufficient to fully satisfy the fatty acid requirements for about 2 million CHO cells per mL.

2.2. CHO cells rapidly take up fatty acids delivered with methyl-β-cyclodextrin

To evaluate how efficiently fatty acids are delivered to CHO cells using MBCD, batch CHO cell cultures were performed, where the cell culture medium was supplemented with fatty acid complexed with 0.05 % MBCD. Five different fatty acids were used: 75 μM [U-^{13}C]palmitic acid (C16:0), 37 μM [U-^{13}C]stearic acid (C18:0; lower concentration of stearic acid was used due to its much lower solubility), 75 μM [U-^{13}C] palmitoleic acid (C16:1), 75 μM [U-^{13}C]oleic acid (C18:1), and 75 μM [U-^{13}C]linoleic acid (C18:2). By using ^{13}C-labeled fatty acids and measuring ^{13}C-labeling of fatty acids in CHO cell mass using gas chromatography-mass spectrometry (GC-MS) we were able to track the metabolic fate of the supplemented fatty acids, i.e. how much and when the supplemented fatty acids were taken up and metabolized by CHO cells.

Fig. 2 shows the growth data and measured ^{13}C-labeling of CHO cell fatty acids for the batch cultures. No growth differences were observed for cultures supplemented with C16:0, C18:0 and C16:1 fatty acids compared to control cultures without fatty acid additions (Fig. 2A). A

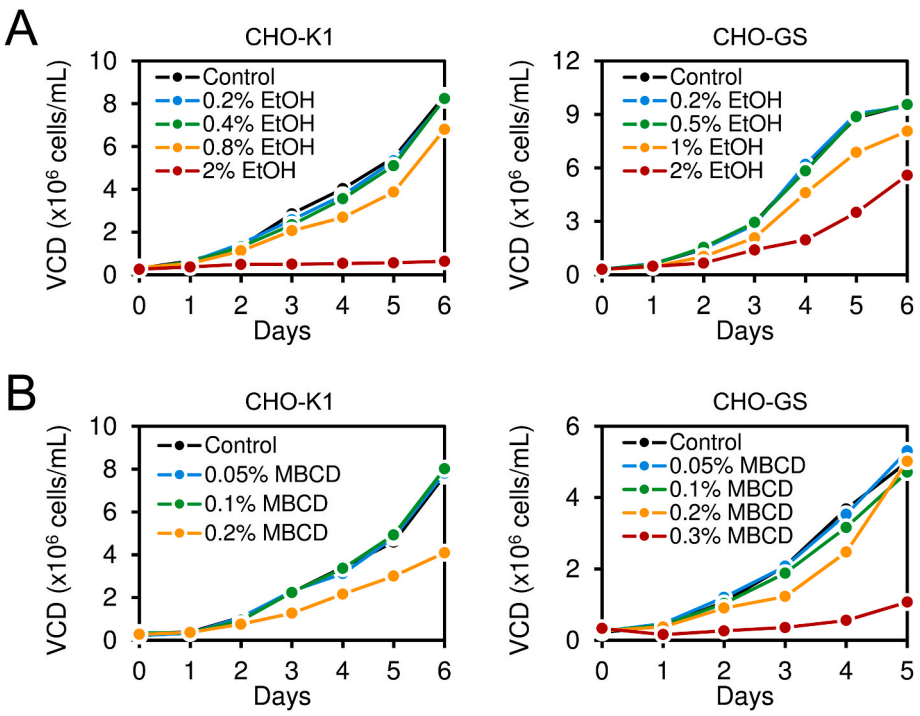

Fig. 1. Determining toxic levels of ethanol and MBCD in CHO cell **cultures.** viable cell densities (VCD) of CHO-K1 cells and CHO-GS cells were determined in batch cultures in media supplemented with different levels of ethanol (**A**) and MBCD (**B**).

slight delay in growth was observed for cultures supplemented with C18:1 and C18:2 (Fig. 2A). As negative controls, we also performed CHO cell cultures with the same ^{13}C-labeled fatty acids added directly into the cell culture medium, that is, without complexation with MBCD. None of these cultures grew well and no ^{13}C-labeling was detected in CHO cells for these cultures. This suggests that complexation is strictly necessary for efficient delivery of fatty acids to CHO cells and that presence of uncomplexed fatty acids in the medium is detrimental to CHO cells.

Significant ^{13}C-labeling was observed in CHO cells for 4 out of 5 fatty acids supplements (Fig. 2B). For all cultures, the highest ^{13}C-labeling was detected for the first sample collected, i.e. on day 1. As an example, for [U-^{13}C]linoleic acid (C18:2), nearly 100 % ^{13}C-labeling was observed for this fatty acid in CHO cell mass on day 1. This is perhaps not surprising since C18:2 is an essential fatty acid that cannot be synthesized *de novo* by CHO cells, i.e. CHO cells do not possess Δ12 desaturase 2 (FAD2) needed to synthesize linoleic acid from oleic acid. Thus, the only source is externally provided linoleic acid. After day 1, ^{13}C-labeling of C18:2 decreased to about 87 % suggesting that another source of C18:2 was present in the medium that was consumed by CHO cells after day 1. Many CHO cell culture media formulations contain undisclosed amounts of linoleic acid. It is likely that the specific cell culture medium used in this study contained some amount of linoleic acid.

The next highest labeling was observed for the culture supplemented with [U-^{13}C]palmitoleic acid (C16:1). On day 1, ^{13}C-labeling of C16:1 in CHO cell mass was 93 %. Interestingly, 37 % ^{13}C-labeling was also observed for C18:1 in this culture (Fig. 2B), suggesting that a fraction of [U-^{13}C]palmitoleic acid was also converted to C18:1 by elongation with one unlabeled AcCoA, i.e. the C18:1 fatty acid was M+16 labeled. After day 1, the ^{13}C-labeling of C16:1 and C18:1 fatty acids in CHO cells steadily decreased to 20 % or less, indicating that after the first day, these fatty acids were synthesized *de novo* from unlabeled AcCoA, such as AcCoA derived from glucose.

A similar pattern was observed for the culture supplemented with [U-^{13}C]oleic acid (C18:1). On day 1, ^{13}C-labeling of C18:1 in CHO cell mass was 59 %. Interestingly, 25 % ^{13}C-labeling was observed for C16:1 in this culture (Fig. 2B), suggesting that a fraction of [U-^{13}C]oleic acid was converted by CHO cells to C16:1 via partial β-oxidation. For the culture supplemented with [U-^{13}C]palmitic acid (C16:0), lower overall labeling was observed in CHO cell fatty acids, with the highest labeling on day 1 reaching only about 40 %. For this culture, all non-essential fatty acids became ^{13}C-labeled (Fig. 2B), suggesting that a large fraction of the externally provided [U-^{13}C]palmitic acid was taken up by CHO cells and further metabolized to C18:0 (by elongase) and to C16:1 and C18:1 (by desaturases). Finally, little or no ^{13}C-labeling was observed for the culture supplemented with [U-^{13}C]stearic acid (C18:0), suggesting that this fatty acid was not taken up and/or metabolized by CHO cells in our cultures (Fig. 2B).

2.3. *A new gas chromatography-mass spectrometry method to quantify fatty acid compositions*

In addition to measuring ^{13}C-labeling of fatty acids, we also wanted to measure relative abundances of fatty acids in CHO cell mass. The most common approach for quantifying the composition of fatty acids in biological samples is to convert the fatty acids into fatty acid methyl esters (FAMEs) and then use gas chromatography with flame ionization detection (GC-FID) for quantification. Here, we wanted to develop an alternative dual-purpose GC-MS based approach that could be used for accurate fatty acid composition analysis as well as ^{13}C-labeling analysis. The key challenge when using GC-MS for quantifying fatty acid levels is that FAMEs of different fatty acids have different ionization efficiencies and as a result the peak intensities do not correlate with relative amounts of the different fatty acids in a sample. For example, we observed that ion counts for unsaturated fatty acids were often 5- to 10-fold lower compared to ion counts for saturated fatty acids present at the same level when the molecular ion was used for quantification (Supplemental Fig. S1A). We observed the same problem when the characteristic McLafferty ion at *m/z* 74 (McLafferty, 1959) was used for quantification (Supplemental Fig. S1B). To solve this problem, we performed full scan GC-MS analysis using a mixture of FAMEs (Supelco 37 FAME mix) and investigated if other ions would better correlate with fatty acid concentrations. The best correlation ($R^2 > 0.98$) was observed

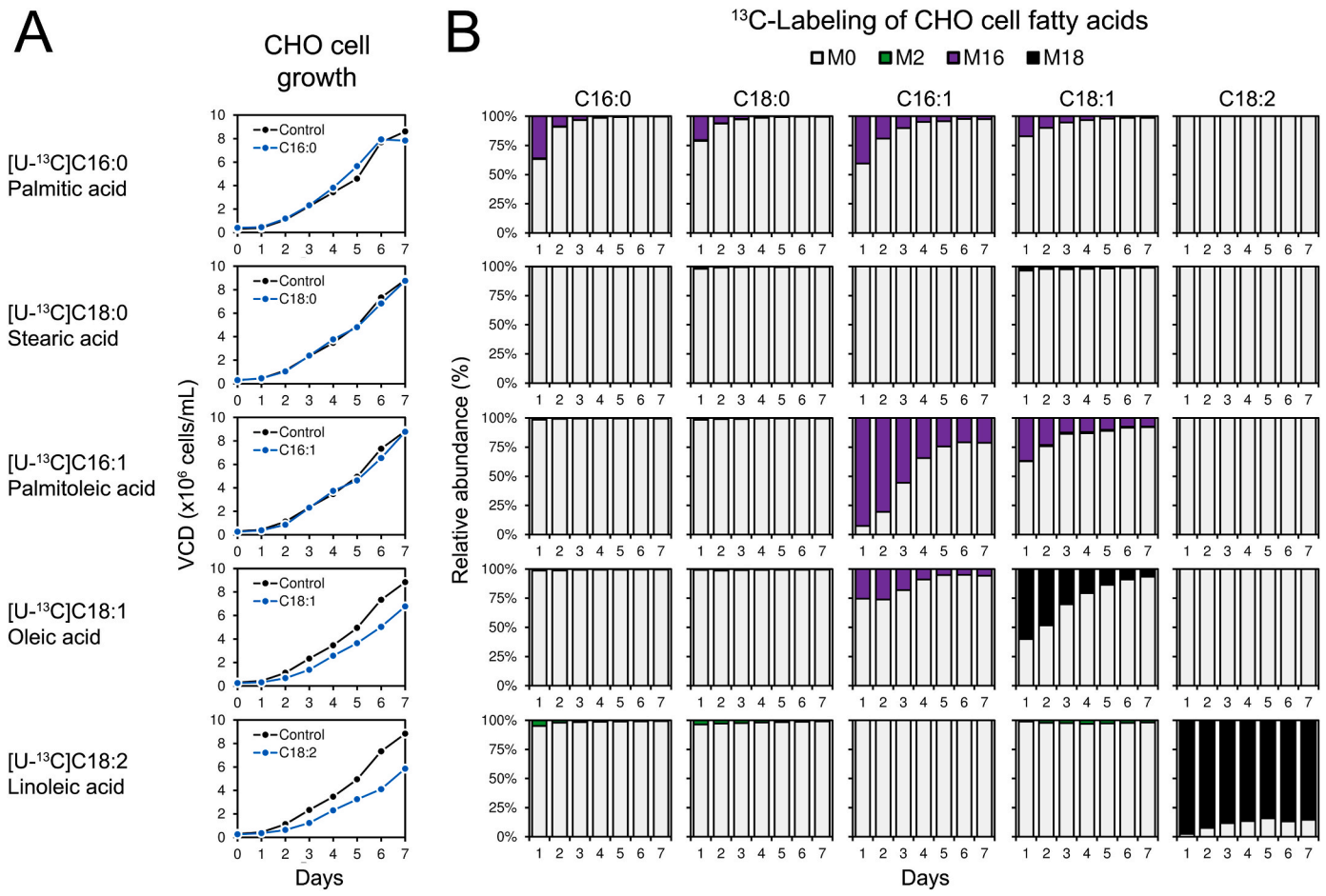

Fig. 2. CHO cells rapidly take up fatty acids delivered with methyl-β-cyc**lodextrin.** CHO-K1 cells were grown in medium containing 0.05 % MBCD and 75 μM of a fully ^{13}C-labeled fatty acid (37 μM was used for stearic acid C18:0 due to lower solubility). Control cultures were inoculated from the same pre-culture and did not receive fatty acids. Shown are viable cell densities (VCD) (**A**), and mass isotopomer distributions of CHO cell fatty acids (**B**) on each day.

when we used the total area under the curve for the ions at *m/z* 59 and 60 (Fig. 3A and B). These two ions appeared in both unlabeled and ^{13}C-labeled fatty acid samples, suggesting that these ions were formed from the methyl group of the fatty acid methyl esters. By further comparing results obtained using the new GC-MS approach to GC-FID we verified that GC-MS could be used to accurately quantify fatty acid

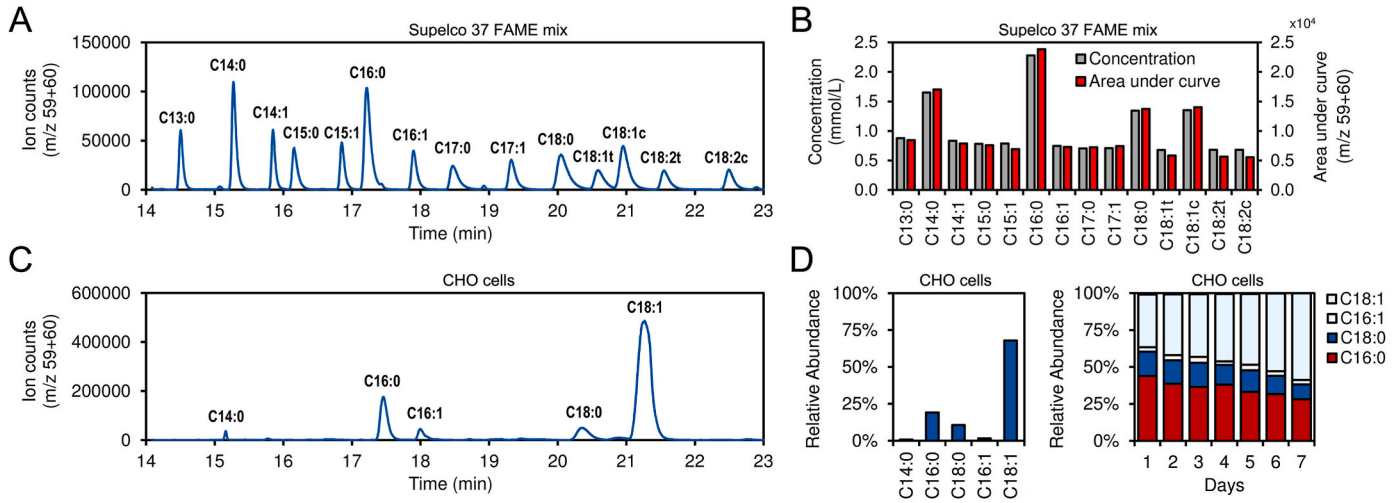

Fig. 3. A new gas chromatography-mass spectrometry method to quantify fatty acid compositions of **CHO cells.** (**A**) A standard mixture of FAMEs with known concentrations (Supelco 37 FAME mix) was analyzed by GC-MS. Shown is the ion chromatogram (for ions at *m/z* 59 and 60) from GC-MS analysis of the Supelco 37 FAME mix. (**B**) The integrated areas under the curve (for ions at *m/z* 59 + 60) mirrored the relative abundances of fatty acids in the Supelco 37 FAME mix. (**C**) Ion chromatogram (for ions at *m/z* 59 and 60) from GC-MS analysis of CHO-K1 cell fatty acids. (**D**) Relative abundances of fatty acids in CHO-K1 cells on different days in a batch culture.

compositions in samples regardless of how much ^{13}C-labeling was present.

We applied this new GC-MS approach in the remainder of this study to quantify fatty acid compositions of CHO cells. As an example, Fig. 3C shows the m/z 59 + 60 ion chromatogram for fatty acid analysis of CHO-K1 cells. Fig. 3D shows the corresponding relative abundances of the fatty acids. Interestingly, when we measured fatty acid compositions of CHO cells at different days in a batch culture we observed significant shifts in the relative abundances of fatty acids (Fig. 3D). Specifically, the relative abundances of saturated fatty acids decreased, i.e. from 43 % to 28 % for C16:0, and from 16 % to 10 % for C18:0, while the relative abundance of the unsaturated fatty acid C18:1 increased from 35 % to 57 %. These results demonstrate that the fatty acid composition of CHO cells is not a constant property of cells, but can change significantly during the course of a typical cell culture. To evaluate if these changes were the result of pool size changes, we also quantified absolute fatty acid levels in CHO cells by using an internal ^{13}C-labeled standard for quantification as described previously (Oates and Antoniewicz, 2022). We found that total fatty acids in CHO cells remained relatively constant over time (Supplemental Fig. S2), suggesting that the changes in fatty acids we observed were largely changes in relative abundances rather than changes in absolute pool sizes.

2.4. Daily feeding of fatty acids impacts fatty acid metabolism and fatty acid composition of CHO cells

Next, we performed fed-batch cultures where fatty acids were fed daily, instead of only on day 0. For these feeding experiments, the culture medium on day 0 contained 37 μM of given ^{13}C-labeled fatty acid complexed with 0.05 % MBCD, and starting on day 1, an additional 37 μM of the same ^{13}C-labeled fatty acid was added directly into the cell culture using a 40 g/L fatty acid stock solution in ethanol; a 20 g/L stock solution was used for stearic acid C18:0 due to its lower solubility. No additional MBCD was added to the cultures. The total amount of ethanol added in these fed-batch cultures was less than 0.4 % v/v, which is below ethanol's toxicity level for CHO cells (see section 2.1).

Fig. 4 shows the results for the experiments with CHO-K1 cells, including growth data, ^{13}C-labeling of CHO cell fatty acids, and fatty acid composition of CHO cells. No growth differences were observed for cultures fed with C16:0, C18:0, C16:1 and C18:1 fatty acids compared to control cultures without fatty acid additions; however, for the culture fed [U-^{13}C]linoleic acid (C18:2), slower growth was observed after day 4 compared to the control (Fig. 4A). The ^{13}C-labeling data confirmed that CHO cells consumed fatty acids fed to the cultures, i.e. the ^{13}C-labeling of CHO cell fatty acids remained roughly constant during the culture (Fig. 4B). In fact, for the fed-batch culture fed with [U-^{13}C]stearic acid (C18:0), ^{13}C-labeling increased over the course of the culture. This is in contrast to the batch culture where [U-^{13}C]stearic acid was added only on day 0, where little or no ^{13}C-labeling incorporation was observed (see section 2.2).

^{13}C-Labeling data further indicated that the metabolism of the supplemented C16:0, C16:1 and C18:1 fatty acids was similar to what was observed in batch cultures described in section 2.2. Briefly, [U-^{13}C]palmitic acid (C16:0) was metabolized to C18:0 (by elongase) and further to C16:1 and C18:1 (by desaturase); [U-^{13}C]palmitoleic acid (C16:1) was metabolized to C18:1 (by elongase); and [U-^{13}C]oleic acid (C18:1) was metabolized to C16:1 via partial β-oxidation (Fig. 4B). For the culture fed [U-^{13}C]stearic acid (C18:0), we observed that this fatty acid was mainly metabolized to C18:1 (by desaturase), although a small amounts of labeling were also observed in C16:1 (up to 12 %) and C16:0 (up to 5 %), suggesting partial β-oxidation. For the fed-batch culture with the essential fatty acid [U-^{13}C]linoleic acid (C18:2), we observed significant M+2 labeling in C16:0, C18:0, C16:1 and C18:2 (up to 17 %, Fig. 4B). This suggests that a significant portion of [U-^{13}C]linoleic acid was catabolized by CHO cells via β-oxidation to form M+2 labeled AcCoA, which was then used for *de novo* biosynthesis of non-essential

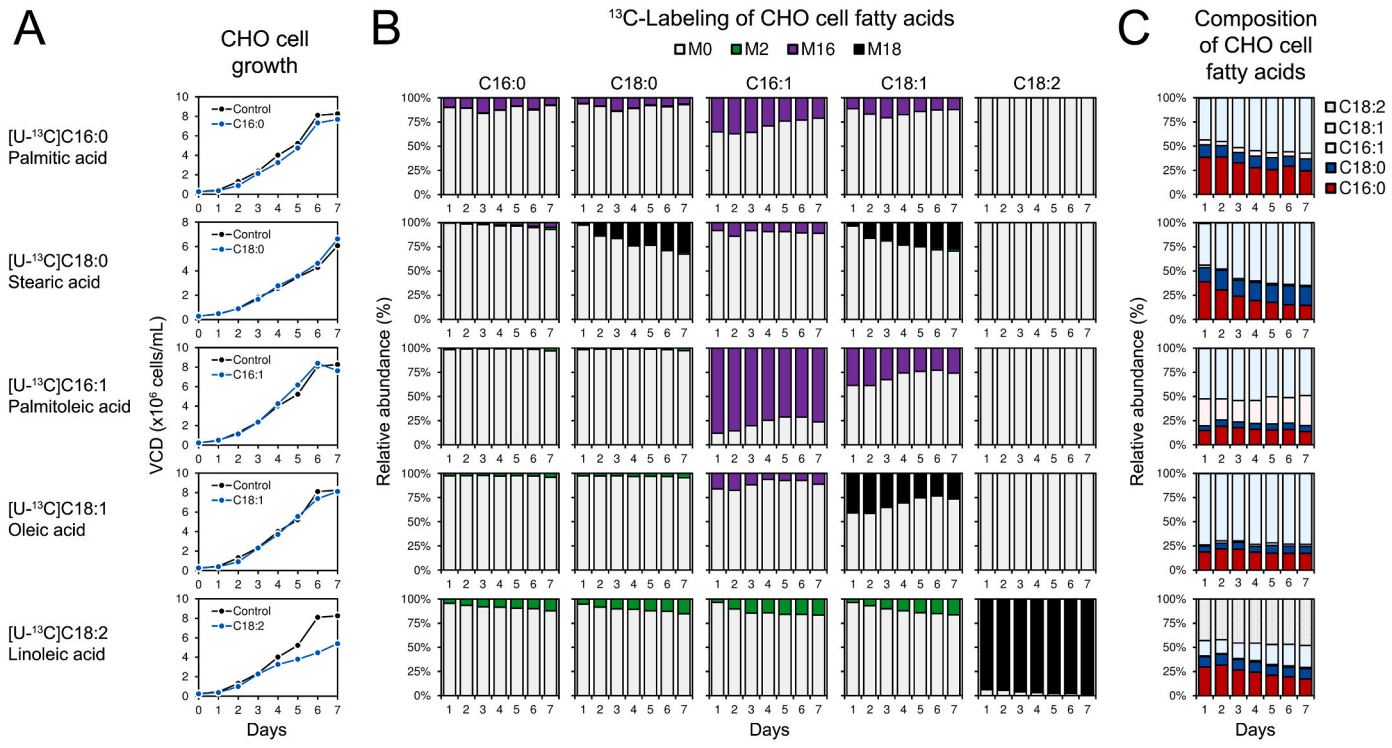

Fig. 4. Fed-batch CHO-K1 cultures with daily feeding of fully ^{13}C-labeled fatty acids (37 μM/day) delivered using methyl-β-cyc**lodextrin.** CHO-K1 cells were grown in medium that contained 0.05 % MBCD and 37 μM of a fully ^{13}C-labeled fatty acid on day 0. Starting on day 1, an additional 37 μM of the same ^{13}C-labeled fatty acid was added directly into the cell culture using a 40 g/L fatty acid stock solution in ethanol (a 20 g/L stock solution was used for stearic acid C18:0). Control cultures were started from the same pre-culture and did not receive fatty acids. Shown are the viable cell densities (VCD) (**A**), mass isotopomer distributions of CHO cell fatty acids (**B**), and fatty acid compositions of CHO cells (**C**) on each day.

fatty acids.

Fig. 4C shows the measured fatty acid compositions of CHO cells for all fed-batch cultures. Interestingly, for the cultures fed the saturated fatty acids C16:0 and C18:0, the fatty acid composition of CHO cells was very similar to the control experiment without fatty acid additions (Fig. 3D), with C16:0 being the dominant fatty acid at the beginning of the culture and C18:1 becoming the dominant fatty acid at the end of the culture. For the culture fed C18:0, the fraction of C18:0 in the biomass was slightly elevated (18 %) compared to the control experiment (14 %). However, the most dramatic changes in fatty acid composition were observed for the cultures fed with unsaturated fatty acids. Significantly, abundances of unsaturated fatty acids were significantly elevated and abundances of saturated fatty acids were significantly reduced; for example, the fraction of C16:0 was below 18 % (compared to 40 % in control culture), and the fraction of C18:0 was below 7 %. For the culture fed C16:1, the fraction of C16:1 in CHO cells increased to 26 % compared to 3 % in the control culture; and for the culture fed C18:1, the C18:1 fatty acid was the main fatty acid in CHO cells throughout the culture with 71 % relative abundance. Finally, for the culture fed with the essential fatty acid linoleic acid (C18:2), the fraction of C18:2 in CHO cell mass increased to 46 % compared to less than 1 % for the control culture. Thus, nearly half of all fatty acids in CHO cell mass were displaced by linoleic acid.

The same fed-batch cultures were also performed with a CHO-GS cell line. The results for these experiments are shown in Supplemental Fig. S3. Overall, the results for the two cell lines were very similar. No growth inhibition was observed for any CHO-GS fed-batch cultures. ^{13}C-Labeling was generally slightly lower for CHO-GS cells (Supplemental Fig. S3B) compared to CHO-K1 cells (Fig. 4B). This was likely due to the higher specific growth rate of CHO-GS cells, i.e. CHO-GS cultures reached peak cell density on day 5 (Supplemental Fig. S3A), compared to day 7 for CHO-K1 cultures (Fig. 4A). The fatty acid compositions for CHO-GS cells (Supplemental Fig. S3C) mirrored the results obtained for CHO-K1 cells (Fig. 4C), with the one notable difference being the fed-batch culture with [U-^{13}C]linoleic acid (C18:2) feeding, which produced a lower abundance of C18:2 in CHO-GS cells (28 %) compared to CHO-K1 cells (46 %). Again, this was likely due to the lower per-cell feed of linoleic acid to CHO-GS cells given their higher growth rate. The reduced growth rate and increased incorporation of C18:2 observed for CHO-K1 cells suggests a potential negative effect of overfeeding this fatty acid to CHO-K1 cells, which was not observed for CHO-GS cells.

To further explore the impact of fatty acid feeding on protein production and metabolism, we also measured extracellular IgG, glucose uptake, glutamine uptake and lactate concentrations for these cultures. The results are summarized in Supplemental Fig. S4. In short, we observed comparable recombinant antibody titers across the different feeding conditions. We also observed similar glucose, lactate and glutamine profiles. This suggests that feeding high levels of fatty acids using MBCD does not have a negative impact on cellular metabolism.

2.5. Methyl-β-cyclodextrin delivers fatty acids to CHO cells as efficiently as bovine serum albumin

To benchmark the efficiency of using MBCD for fatty acid delivery to CHO cells, we repeated the same feeding experiments as described in the previous section, with the only difference being that 0.2 % w/v bovine serum albumin (BSA) was used as the fatty acid carrier instead of 0.05 % w/v MBCD. The results for these experiments are shown in Supplemental Fig. S5 for CHO-K1 cells, and Supplemental Fig. S6 for CHO-GS cells. Overall, the results for the experiments with BSA were very similar to the results obtained with MBCD. A few notable differences included the following. First, we observed improved growth for BSA containing cultures compared to control cultures for CHO-K1 cells. This suggests that the BSA used here may have contained some unknown growth enhancing components. Second, in a few cases, increased ^{13}C-labeling in CHO cell fatty acids was observed for experiments with BSA compared to MBCD (Supplemental Fig. S7). For example, slightly higher labeling was observed for [U-^{13}C]palmitic acid complexed with BSA compared to MBCD for CHO-K1 cells, and slightly higher labeling was observed for [U-^{13}C]palmitoleic acid complexed with BSA compared to MBCD for CHO-GS cells (Supplemental Fig. S7). Taken together, however, these results indicate that MBCD delivers fatty acids to CHO cells very efficiently and should be considered the preferred choice for this culture system, given that it is not animal-derived, is more cost effective, and can be implemented in chemically defined media formulations.

2.6. Fatty acid composition of CHO cells is controlled with relatively modest fatty acid feeding

Fig. 5 summarizes the results for all fed-batch cultures performed in this study, that is, using two different CHO cell lines (i.e. CHO-K1 and CHO-GS cells), five different fatty acid supplements, and two different delivery methods (i.e. MBCD and BSA). Using the measured fatty acid composition data and ^{13}C-labeling data, we calculated the total percentage of fatty acids that originated from the supplemented fatty acids for each experiment, and determined the relative degree of saturation of CHO cell fatty acids, i.e. the ratio of saturated vs. mono-unsaturated vs. poly-unsaturated fatty acids (S:M:P ratio), which are also shown in Fig. 5.

When comparing the two CHO cell lines, we observed that CHO-K1 cells incorporated higher amounts (i.e. 60 %–90 % more) of the supplemented fatty acids into cell mass than CHO-GS cells. As discussed in section 2.4, this is the result of the higher per-cell feeding of fatty acids to CHO-K1 cells compared to CHO-GS cells, i.e. the same amount of fatty acids was supplemented daily to all cultures, but CHO-K1 cells grew slower than CHO-GS cells. Regardless of this, for both CHO cell lines, only a relatively small faction of cellular fatty acids originated from the supplemented fatty acids, ranging from 14 % to 43 % for CHO-K1 cells, and from 6 % to 26 % for CHO-GS cells. The remaining fraction of fatty acids was synthesized *de novo*. These percentages can be explained by considering the amount of fatty acids fed daily to the cultures, i.e. 37 μM/day. In theory, feeding 37 μM/day of fatty acids daily could satisfy the fatty acid requirements for about 0.5 million CHO cells per mL per day (see section 2.1). However, during exponential growth, we often observed net growth of 1–3 million CHO cells per mL per day. This explains why the majority of fatty acids were still synthesized *de novo* by CHO cells.

When looking at the fatty acid compositions of CHO cells, even using a 37 μM/day feeding rate resulted in dramatic changes in the degree of saturation in CHO cell fatty acids. For control cultures, the ratio of saturated vs. mono-unsaturated vs. poly-unsaturated fatty acids (S:M:P ratio) was about 50:50:0 for both CHO cell lines. Interestingly, for cultures fed with saturated fatty acids, i.e. C16:0 and C18:0, the degree of saturation did not increase; in fact, it decreased slightly to a S:M:P ratio of approximately 46:54:0. This suggests that CHO cells predominantly metabolized saturated fatty acids further to unsaturated fatty acids as part of their normal metabolism. This was further supported by the ^{13}C-labeling data. For example, for tracer experiments with [U-^{13}C] palmitic acid, a higher ^{13}C-labeling was observed for C16:1 and C18:1 fatty acids than for C16:0 and C18:0 fatty acids (Fig. 5).

For cultures fed with mono-unsaturated fatty acids, i.e. C16:1 and C18:1, the degree of saturation in CHO cell fatty acids decreased significantly for both CHO cell lines, resulting in a S:M:P ratio of about 25:75:0. Moreover, no ^{13}C-labeling was observed in the saturated fatty acids (Fig. 5), indicating that unsaturated fatty acids were not converted to saturated fatty acids, consistent with the current understanding of mammalian fatty acid biochemistry, i.e. there are no known fatty acid hydrogenation enzymes. Finally, for cultures fed with the poly-unsaturated fatty acid linoleic acid (C18:2), the S:M:P ratio was 35:20:45 for CHO-K1 cells and 45:29:26 for CHO-GS cells. These results suggest that linoleic acid may have an inhibitory effect on the activity of fatty acid desaturases, since for both cell lines we observed that the

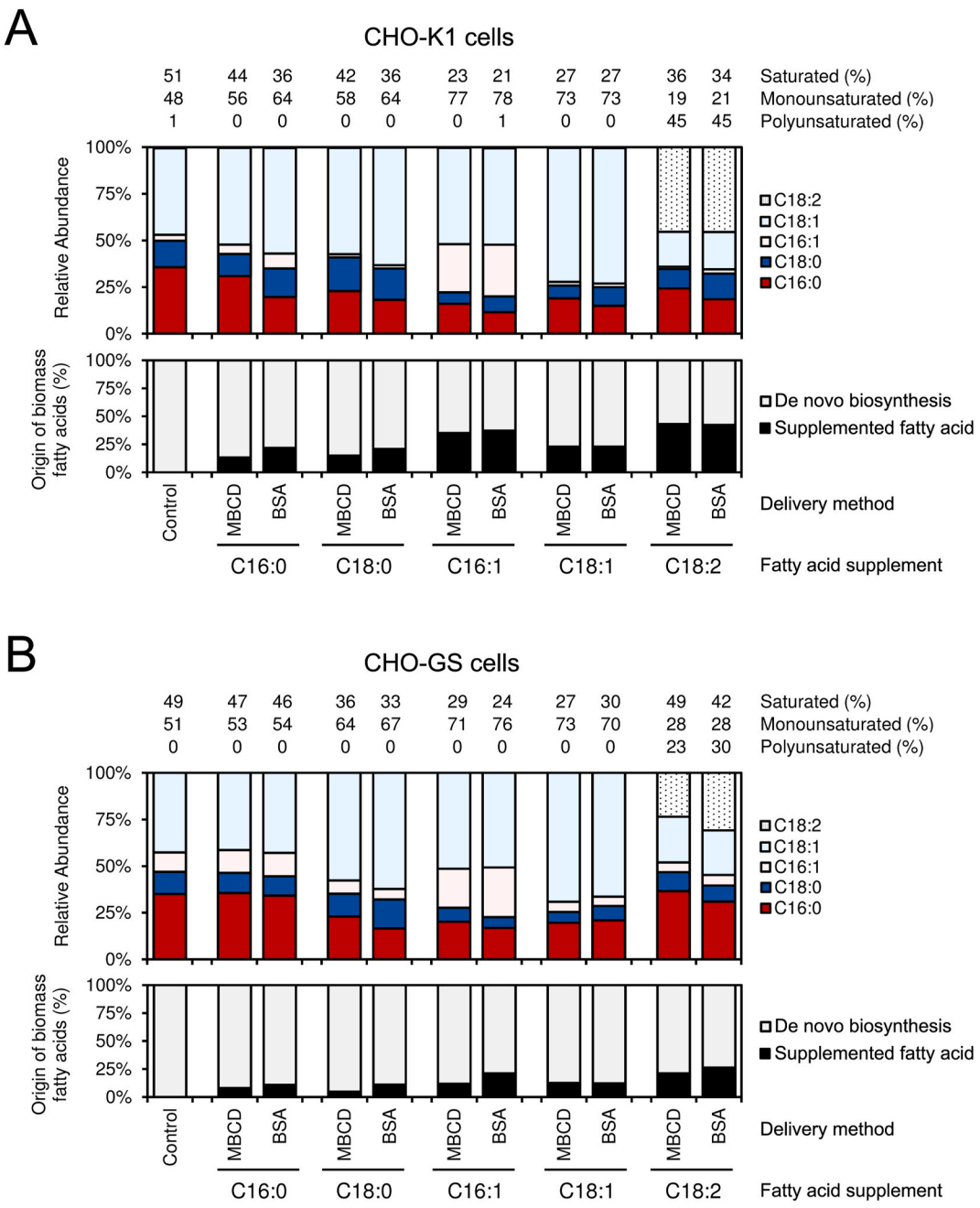

Fig. 5. Fatty acid analysis for fed-batch cultures performed using two different CHO cell lines, five different fatty acid supplements, and two different delivery methods. Relative abundances of saturated, monounsaturated and polyunsaturated fatty acids, and the percentage of fatty acids that originated from the supplemented fatty acids were calculated from the measured fatty acid compositions and ^{13}C-labeling data. All data shown are averaged values over the duration of the fed-batch cultures for CHO-K1 (**A**) and CHO-GS cells (**B**).

fraction of saturated fatty acids was higher than the fraction of monounsaturated fatty acids, with a S:M ratio of about 3:2, compared to 1:1 for control cultures, and 1:3 for cultures fed with unsaturated fatty acids.

Finally, we evaluated the impact of using even higher fatty acid feeding rates in CHO cell cultures, which we expected would further reduce the requirement for *de novo* fatty acid biosynthesis and potentially further alter fatty acid abundances. Specifically, we repeated feeding experiments using both CHO cell lines with MBCD added on day 0, but now using 75 μM daily feeding of [U-^{13}C]palmitic acid (C16:0) or [U-^{13}C]palmitoleic acid (C16:1) instead of 37 μM daily feeding. The results of these experiments are summarized in Fig. 6, Supplemental Fig. S8 and Supplemental Fig. S9. As expected, we observed significantly higher ^{13}C-labeling incorporation and further modulation of relative fatty acid abundances. In particular, we observed significantly increased abundances of C16:1 in all experiments. For example, for CHO-K1 cells fed with [U-^{13}C]palmitoleic acid, C16:1 became the dominant fatty acid. The degree of saturation also further decreased, reaching a S:M:P ratio of approximately 16:84:0 for both CHO cell lines with 75 μM daily feeding of [U-^{13}C]palmitoleic acid. *De novo* biosynthesis decreased as expected (Fig. 6). For both CHO cell lines, the fraction of cellular fatty acids derived from the supplemented fatty acid roughly doubled with 75 μM daily feeding compared to 37 μM daily feeding. For example, for CHO-K1 cells, the fraction of cellular fatty acids derived from the supplemented [U-^{13}C]palmitoleic acid increased from 34 % to 67 %. Taken together, these data demonstrate that feeding fatty acids in CHO cell cultures is an effective strategy to modulate relative abundances of fatty

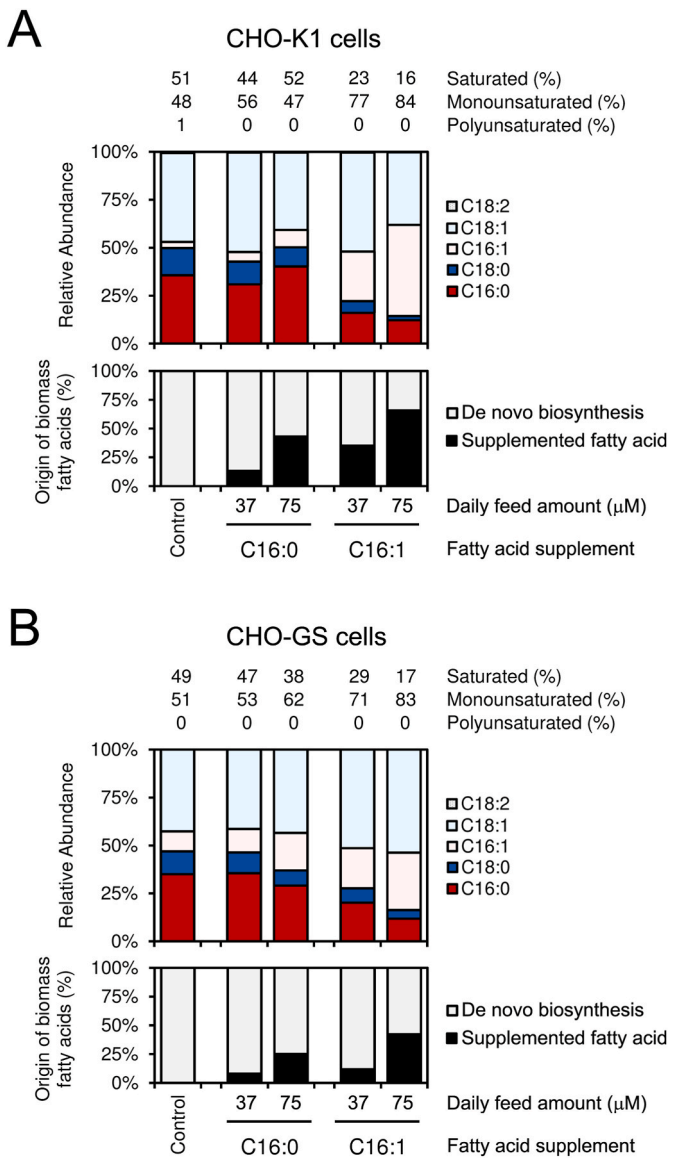

Fig. 6. Fatty acid analysis for fed-batch cultures performed using two different CHO cell lines with either 37 μM/day or 75 μM/day feeding of [U-^{13}C]palmitic acid or [U-^{13}C]palmito**leic aicd.** Relative abundances of saturated, monounsaturated and polyunsaturated fatty acids, and the percentage of fatty acids that originated from the supplemented fatty acids were calculated from the measured fatty acid compositions and ^{13}C-labeling data. All data shown are averaged values over the duration of the fed-batch cultures for CHO-K1 (**A**) and CHO-GS cells (**B**).

acids, the degree of saturation of fatty acids, and *de novo* biosynthesis of fatty acids in CHO cells.

2.7. CHO cells metabolize unsaturated fatty acids to less common fatty acids: vaccenic acid and hypogeic acid

Fig. 7 shows the canonical biochemical pathways for *de novo* fatty acid biosynthesis in CHO cells (green arrows in Fig. 7). Glucose is the main carbon source for *de novo* fatty acid biosynthesis (Ahn and Antoniewicz, 2013; Ahn et al., 2016). Glucose is first metabolized via glycolysis to form AcCoA, which then undergoes condensation through the enzymatic actions of acetyl-CoA carboxylase and fatty acid synthase (FASN) to produce palmitic acid. All other non-essential fatty acids are then produced from palmitic acid through the enzymatic actions of fatty acid elongases (ELOVL1-7), Δ9 stearoyl-CoA desaturases 1 and 5 (SCD1, SCD5), and fatty acid desaturases 1 and 2 (FADS1, FADS2) (Alcoriza-Balaguer et al., 2023).

Interestingly, ^{13}C-labeling data in this study suggest that at least two additional less common fatty acids were synthesized by CHO cells from the supplemented fatty acids (Fig. 7, red arrows). First, in tracer experiments with [U-^{13}C]palmitoleic acid (C16:1n-7) we observed ^{13}C-labeling in C18:1. This C18:1 fatty acid was likely not the common fatty acid oleic acid (C18:1n-9), since there is no known pathway for converting palmitoleic acid to oleic acid. Instead, the C18:1 fatty acid was likely vaccenic acid (C18:1n-7), which can be produced from palmitoleic acid via the action of elongases ELOVL1,3,7 (Alcoriza-Balaguer et al., 2023; Bermudez et al., 2022). Similarly, in tracer experiments with [U-^{13}C]oleic acid (C18:1n-9) we observed ^{13}C-labeling in C16:1. This C16:1 fatty acid was likely not palmitoleic acid (C16:1n-7), since there is no known pathway for converting oleic acid to palmitoleic acid. Instead, the C16:1 fatty acid was likely hypogeic acid (C16:1n-9), which can be produced from oleic acid via partial β-oxidation (Bermudez et al., 2022). Based on the measured fatty acid compositions and ^{13}C-labeling data, we estimated the relative abundances of the two less common fatty acids in CHO cells. For fed-batch cultures with oleic acid feeding, the abundance of hypogeic acid in CHO cell mass was very small, less than 5 %, for both CHO cell lines. However, for fed-batch cultures with palmitoleic acid feeding, the abundance of vaccenic acid in CHO cell mass was significant: at least 15 % for CHO-K1 cells and at least 10 % for CHO-GS cells.

To provide additional support for the presence of vaccenic acid and hypogeic acid in CHO cells, we optimized a GC-MS protocol for detecting these fatty acids independent of the more common fatty acids palmitoleic acid and oleic acid. First, we supplemented the Supelco 37 FAME mixture with either hypogeic acid or vaccenic acid and analyzed it by GC-MS. Fig. 8A shows that the different fatty acids can be individually quantified as they elute at different times, i.e. hypogeic acid (C16:1n-9) elutes at 15.62min, palmitoleic acid (C16:1n-7) elutes at 15.67min, oleic acid (C18:1n-9) elutes at 17.54min, and vaccenic acid (C18:1n-7) elutes at 17.60min. We then applied this GC-MS protocol to re-analyze samples from our fed-batch studies with CHO-GS cells. In Fig. 8B, it can be seen that hypogeic acid was present at very low levels in all experiments (<1 % relative abundance), except in the experiment with oleic acid feeding (~5 %). This is consistent with our ^{13}C-labeling data and the proposed pathway for hypogeic acid biosynthesis. Similarly, we observed that vaccenic acid was present at relatively low levels (<15 %) in all experiments, except in the experiment with palmitoleic acid feeding, where it became a dominant fatty acid in CHO cells. This is again consistent with our ^{13}C-labeling data and the proposed pathway for vaccenic acid biosynthesis.

Thus far, relatively little is known about the roles and effects of hypogeic acid and vaccenic acid in mammalian biology. Hypogeic acid is normally not present at high levels in biological systems, and its role has been studied mostly in the context of cardiovascular disease, with elevated levels appearing to serve as a biomarker for cardiovascular damage, and potentially playing a role in mediating the anti-inflammatory cellular response (Bermudez et al., 2022). Vaccenic acid, on the other hand, is known to be present at relatively high levels in milk, although its role as a nutrient is still unclear (Field et al., 2009). Vaccenic acid has been reported to decrease growth of cancer cells, as well as protect humans against coronary heart disease (Aimola et al., 2016). Finally, vaccenic acid was shown to modulate the mTORC2-Akt-FoxO1 pathway involved in differentiation (Aimola et al., 2016), and was suggested to have anti-oxidant and anti-inflammatory properties (Aimola et al., 2016; Badawy et al., 2023). To date, neither hypogeic acid nor vaccenic acid have been investigated in the context of CHO cell cultures. Further research is needed to better understand the roles and potential uses of these two fatty acids.

3. Conclusions

In this study, we have used fatty acid feeding with methyl-

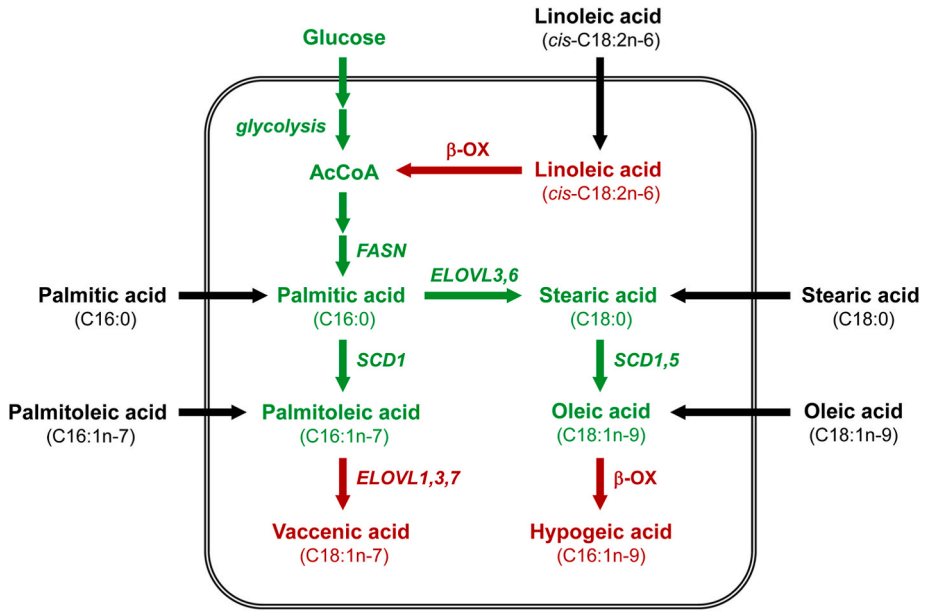

Fig. 7. Overview of fatty acids metabolism in **CHO cells.** *De novo* biosynthesis of fatty acids from glucose is represented by green arrows. Black arrows represent uptake of supplemented fatty acids. Red arrows represent metabolism of supplemented fatty acids as elucidated in this study using ^{13}C-tracing. Abbreviations: fatty acid synthase (FASN); fatty acid elongases (ELOVL), Δ9 stearoyl-CoA desaturases (SCD); β-oxidation (β-OX). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

β-cyclodextrin as the delivery vehicle to modulate fatty acid composition of CHO cells. Additionally, we applied ^{13}C-tracing to elucidate the underlying changes in fatty acid metabolism. We specifically focused on C16 and C18 fatty acids, since these are the dominant fatty acids in CHO cells (Subbaiah et al., 2011). It is known that CHO cells can also uptake very long chain fatty acids (VLCFAs) (Ali et al., 2022). This could be a focus of future investigations using the techniques developed in this study. It is important to note that with our FAME derivatization protocol we were only able to measure saponifiable fatty acids, such as free fatty acids and fatty acids that comprise fatty acid esters, such as phospholipids, diacylglycerides, and triacylglycerides. While these saponifiable fatty acids comprise the majority of fatty acid-containing lipids in CHO cells, there are some lipids that we are unable to detect, such as sphingolipids. Lipidomic characterization of CHO cells performed by Szeliova et al. however, demonstrated that the vast majority of lipids in CHO cells are saponifiable (Szeliova et al., 2020). Rivier et al. estimated that the ratio of phospholipids (saponifiable) to sphingolipids (not saponifiable) is approximately 85:15 in mammalian cells (Rivier et al., 2010).

Feeding unsaturated fatty acids such as palmitoleic acid, oleic acid, and linoleic acid had the greatest impact the fatty acid composition of CHO cells in this study, increasing their respective abundances in cell mass by upwards of 25x, 1.5x, and 50x, respectively. ^{13}C-Tracing revealed that supplemented fatty acids were involved in a range of elongation, desaturation, and β-oxidation reactions to yield common and uncommon fatty acids. In particular, monounsaturated fatty acids were metabolized to at least two uncommon fatty acids, i.e. palmitoleic acid was elongated to generate vaccenic acid, and oleic acid underwent partial β-oxidation to generate hypogeic acid. The impacts of these fatty acids have yet to be characterized in CHO cells.

This study further established that CHO-K1 and CHO-GS cells can efficiently take up high levels of fatty acids provided to the culture when solubilized with methyl-β-cyclodextrin (MBCD) at rates comparable to delivery using bovine serum albumin (BSA). We believe that the rapid release of fatty acids using the MBCD delivery method will in the future allow better control of fatty acid composition and CHO cell physiology compared to genetic engineering strategies, i.e. supplementation of fatty acids to cell culture media is a simpler approach than genetically engineering CHO cells to elicit changes in fatty acid profiles. The fatty acid delivery method developed here is also cost effective as MBCD is only supplemented on day 0 and we have demonstrated that it is functional to solubilize additionally added fatty acids over the course of a fed batch culture.

In the present study, we only explored delivering a single fatty acid at a time. Future work could focus on investigating and optimizing the delivery of multiple fatty acids and developing methods to direct the metabolic fate of these fatty acids once inside the cell. Interestingly, in this study we did not observe any significant impact on IgG secretion, or consumption and secretion rates of metabolites such as amino acids, glucose, or lactate. In general, the relationship between fatty acid composition in CHO cells and protein secretion is still underexplored. In one study, fatty acid metabolism was altered by changing genetic overexpression of SREBP and SCD1, enzymes involved in unsaturated fatty acid biosynthesis (Budge et al., 2020). While this resulted in increased protein secretion, it also changed other aspects of cell morphology such as vesicle budding characteristics and endoplasmic reticulum expansion. This demonstrates that altered fatty acid compositions could have a much broader impact on CHO cells. Some possible effects could include changes in ER bilayer composition (affecting secretory capacity), plasma membrane bilayer properties, for example, increasing membrane fluidity that could reduce energy needed for vesicle budding (Settles et al., 2010), secondary messenger activity, and signal transduction pathways (Harayama and Antonny, 2023; Maulucci et al., 2016; Ramirez and Mutharasan, 1990). Additional fatty acid feeding experiments are also needed in industrially relevant production formats such as perfusions to further probe the relationship between fatty acid biomass composition and recombinant protein production. In future work, we are planning to investigate the broader effects of fatty acid supplementation on cellular physiology and metabolism using complementary techniques such as transcriptomics, genome scale modeling and ^{13}C metabolic flux analysis (Antoniewicz et al., 2006; Chen et al., 2019; Long and Antoniewicz, 2019).

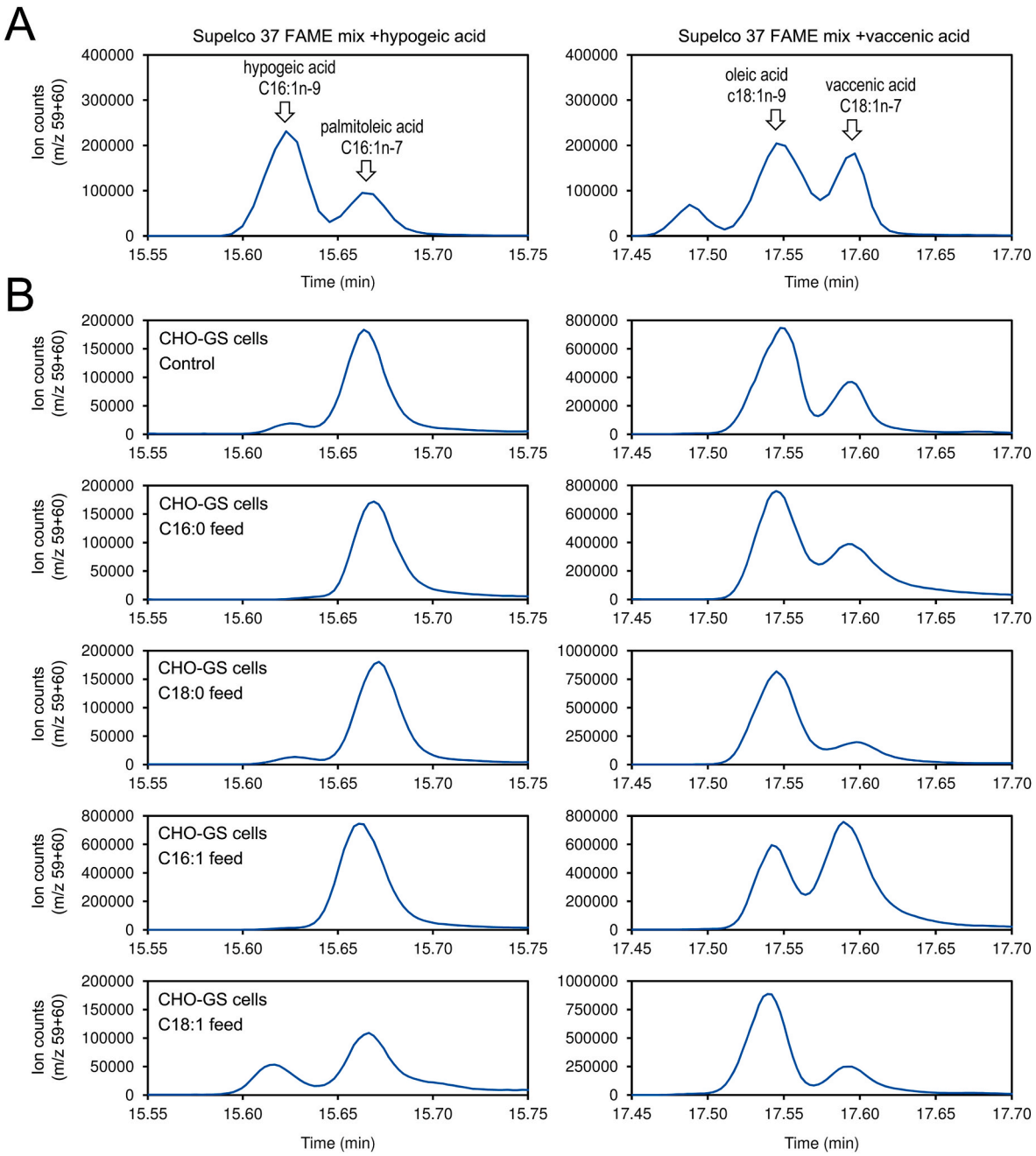

Fig. 8. Quantification of hypogeic, palmitoleic, oleic and vaccenic acids by GC-MS **analysis.** (**A**) Ion chromatograms from GC-MS analysis of Supelco 37 FAME mixture supplemented with either hypogeic acid or vaccenic acid. The four different fatty acids can be individually quantified since they elute at different times when separated on a HP-5MS column. (**B**) Analysis of fatty acids in CHO cells from fed-batch experiments with CHO-GS cells with and without fatty acid feeding. Hypogeic acid level was elevated only in the experiment with oleic acid feeding. Vaccenic acid level was elevated only in the experiment with palmitoleic acid feeding.

4. Materials and methods

4.1. Materials

Fatty acid tracers [U-^{13}C]linoleic acid (Cat No. CLM-6855), [U-^{13}C] stearic acid (Cat No. CLM-6990), and [U-^{13}C]palmitoleic acid (Cat No. CLM-2241) were purchased from Cambridge Isotope Laboratories (Andover, MA). [U-^{13}C]Palmitic acid (Cat No. 605573), [U-^{13}C]oleic acid (Cat No. 490431), unlabeled fatty acids, methyl-β-cyclodextrin (Cat No. C4555), bovine serum albumin (Cat No. A5611 and A1595), Supelco 37 component fatty acid methyl ester (FAME) mixture (Cat No. 47885-U), and chemically defined cell culture media (Immediate Advantage, Cat. No. 87093C) were purchased from MilliporeSigma (Saint Louis, USA). Isotopic purity of all tracers was verified by GC-MS (Long and Antoniewicz, 2019). All media were filter-sterilized prior to use in cell culture experiments using a 0.22 or 0.4 μm pore-size membrane filter.

4.2. Cell culture

Two CHO cell lines were used in this study: a CHO-GS cell line (CHOZN® GS−/− ZFN-modified CHO cell line), and a CHO-K1 cell line (DHFR expression system). Both CHO cell lines produce the antibody VRC01. CHO cells were grown in suspension cultures in a humidified incubator operating at 37 °C, 125 RPM and 5 % CO_2. For the CHO-K1 cell line, 6 mM glutamine and 0.4 vol% anti-clumping agent (Gibco, Canada) were added to the medium. Cell cultures were seeded at 0.3 ×

10^6 cells/mL in 25–30 mL of medium and CHO cells were grown in 125 mL shaker flasks (Corning Erlenmeyer cell culture flasks, Sigma-Aldrich, Cat No. CLS431143). For experiments with daily feeding of fatty acids, culture medium contained 0.3 % v/v Penicillin-Streptomycin solution to minimize the risk of contamination. To obtain cell pellets, a pre-determined amount of cell culture was harvested and centrifuged at 1000 RPM to separate the supernatant from cell pellet. Spent media were stored at −20 °C. Cell pellets were washed once using cold saline solution and stored at −20 °C.

4.3. Delivery of fatty acids in cell culture

To deliver fatty acids on day 0 in cell cultures, fatty acids were first complexed with either methyl-β-cyclodextrin (MBCD) or bovine serum albumin (BSA). Briefly, fatty acids were added to a 20 % solution of MBCD in water (w/v), or a 10 % BSA solution (w/v). The mixtures were heated at 50 °C for at least 30 min with repeated sonication until fatty acids were fully complexed as determined by visual inspection of the solutions. The fatty acid/MBCD (or fatty acid/BSA) solution was then added directly to the culture medium and the medium was filter-sterilized. For daily feeding of fatty acids in CHO cell cultures, fatty acids were dissolved in ethanol at a concentration of 40 g/L, except for stearic acid which was dissolved in ethanol at 20 g/L due to its lower solubility. The ethanol solution was then heated to 37 °C and added directly into the CHO cell culture.

4.4. Analytical methods

Viable cell density (VCD) and cell viability (%) were determined from cell counts based on the trypan blue dye exclusion method using a hemocytometer or automated cell counter (Invitrogen Countess 3) for CHO-GS cells, or an automated cell counter (Corning Cytosmart) for CHO-K1 cells. If cell clumps were visible, culture samples were first mixed with 0.25 % trypsin-EDTA (Gibco, Canada) at 1:1 ratio and incubated for 10 min at 37 °C, prior to cell counting. Glucose and lactate concentrations were measured using a YSI 2950D or YSI 2500 biochemical analyzer (Yellow Spring Instrument). To quantify concentrations of amino acids in spent media, samples were mixed with an internal standard solution containing ^{13}C-labeled amino acids with a known concentration and analyzed by GC-MS (Oates and Antoniewicz, 2023).

4.5. Gas chromatography-mass spectrometry

To quantify fatty acid composition and ^{13}C-labeling of fatty acids in CHO cells by GC-MS, cell pellets containing 1 to 2 million cells were collected, washed using cold saline solution, and fatty acids were extracted using the methanol/chloroform/water extraction method (Crown et al., 2015). Fatty acids were then derivatized to form fatty acid methyl esters (FAME) (Oates and Antoniewicz, 2022) and analyzed by gas chromatography-mass spectrometry (GC-MS). GC-MS analysis was performed on an Agilent 7890A GC system equipped with a Select FAME column (100 m, 0.25 mm i.d., Agilent J&W Scientific) or a HP-5MS column (30 m, 0.25 mm i.d., 0.25 μm-phase thickness; Agilent J&W Scientific), connected to an Agilent 5977B Mass Spectrometer operating under ionization by electron impact (EI) at 70 eV. Helium flow was maintained at 1 mL/min. The source temperature was maintained at 230 °C, the MS quad temperature at 150 °C, the interface temperature at 280 °C, and the inlet temperature at 280 °C. Mass spectra were recorded in single ion monitoring (SIM) mode with 4ms dwell time on each ion. Mass isotopomer distributions were obtained by integration of ion chromatograms (Gomez et al., 2023), and corrected for natural isotope abundances (Fernandez et al., 1996).

CRediT authorship contribution statement

Bradley Priem: Writing – original draft, Validation, Investigation, Formal analysis, Data curation. **Xiangchen Cai:** Writing – original draft, Validation, Investigation, Formal analysis, Data curation. **Yu-Jun Hong:** Validation, Investigation, Formal analysis, Data curation. **Karl Gilmore:** Investigation, Formal analysis. **Zijun Deng:** Investigation, Formal analysis. **Sabrina Chen:** Investigation, Formal analysis. **Harnish Mukesh Naik:** Validation, Methodology. **Michael J. Betenbaugh:** Writing – review & editing, Project administration, Funding acquisition, Data curation, Conceptualization. **Maciek R. Antoniewicz:** Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgements

This work was funded and supported by the Advanced Mammalian Biomanufacturing Innovation Center (AMBIC) through the Industry-University Cooperative Research Center Program under U.S. National Science Foundation grant number 1624684.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymben.2025.04.005.

Data availability

Data will be made available on request.

References

Ahn, W.S., Antoniewicz, M.R., 2013. Parallel labeling experiments with [1,2-(13)C] glucose and [U-(13)C]glutamine provide new insights into CHO cell metabolism. Metab. Eng. 15, 34–47.
Ahn, W.S., Crown, S.B., Antoniewicz, M.R., 2016. Evidence for transketolase-like TKTL1 flux in CHO cells based on parallel labeling experiments and (13)C-metabolic flux analysis. Metab. Eng. 37, 72–78.
Aimola, I.A., Inuwa, H.M., Nok, A.J., Mamman, A.I., Bieker, J.J., 2016. Cis-vaccenic acid induces differentiation and up-regulates gamma globin synthesis in K562, JK1 and transgenic mice erythroid progenitor stem cells. Eur. J. Pharmacol. 776, 9–18.
Alcoriza-Balaguer, M.I., Garcia-Canaveras, J.C., Benet, M., Juan-Vidal, O., Lahoz, A., 2023. FAMetA: a mass isotopologue-based tool for the comprehensive analysis of fatty acid metabolism. Briefings Bioinf. 24.
Ali, A.S., Raju, R., Ray, S., Kshirsagar, R., Gilbert, A., Zang, L., Karger, B.L., 2018. Lipidomics of CHO cell bioprocessing: relation to cell growth and specific productivity of a monoclonal antibody. Biotechnol. J. 13, e1700745.
Ali, H., Morito, K., Hasi, R.Y., Aihara, M., Hayashi, J., Kawakami, R., Kanemaru, K., Tsuchiya, K., Sango, K., Tanaka, T., 2022. Characterization of uptake and metabolism of very long-chain fatty acids in peroxisome-deficient CHO cells. Biochim. Biophys. Acta Mol. Cell Biol. Lipids 1867, 159088.
Antoniewicz, M.R., 2018. A guide to (13)C metabolic flux analysis for the cancer biologist. Exp. Mol. Med. 50, 1–13.
Antoniewicz, M.R., Stephanopoulos, G., Kelleher, J.K., 2006. Evaluation of regression models in metabolic physiology: predicting fluxes from isotopic data without knowledge of the pathway. Metabolomics 2, 41–52.
Badawy, S., Liu, Y., Guo, M., Liu, Z., Xie, C., Marawan, M.A., Ares, I., Lopez-Torres, B., Martinez, M., Maximiliano, J.E., Martinez-Larranaga, M.R., Wang, X., Anadon, A., Martinez, M.A., 2023. Conjugated linoleic acid (CLA) as a functional food: is it beneficial or not? Food Res. Int. 172, 113158.
Bermudez, M.A., Pereira, L., Fraile, C., Valerio, L., Balboa, M.A., Balsinde, J., 2022. Roles of palmitoleic acid and its positional isomers, hypogeic and sapienic acids, in inflammation, metabolic diseases and cancer. Cells 11.
Brunaldi, K., Huang, N., Hamilton, J.A., 2010. Fatty acids are rapidly delivered to and extracted from membranes by methyl-beta-cyclodextrin. J. Lipid Res. 51, 120–131.
Budge, J.D., Knight, T.J., Povey, J., Roobol, J., Brown, I.R., Singh, G., Dean, A., Turner, S., Jaques, C.M., Young, R.J., Racher, A.J., Smales, C.M., 2020. Engineering of Chinese hamster ovary cell lipid metabolism results in an expanded ER and enhanced recombinant biotherapeutic protein production. Metab. Eng. 57, 203–216.
Chen, Y., McConnell, B.O., Gayatri Dhara, V., Mukesh Naik, H., Li, C.T., Antoniewicz, M. R., Betenbaugh, M.J., 2019. An unconventional uptake rate objective function

approach enhances applicability of genome-scale models for mammalian cells. NPJ Syst Biol Appl 5, 25.
Christian, A.E., Haynes, M.P., Phillips, M.C., Rothblat, G.H., 1997. Use of cyclodextrins for manipulating cellular cholesterol content. J. Lipid Res. 38, 2264–2272.
Crown, S.B., Marze, N., Antoniewicz, M.R., 2015. Catabolism of branched chain amino acids contributes significantly to synthesis of odd-chain and even-chain fatty acids in 3T3-L1 adipocytes. PLoS One 10, e0145850.
Fernandez, C.A., Des Rosiers, C., Previs, S.F., David, F., Brunengraber, H., 1996. Correction of 13C mass isotopomer distributions for natural stable isotope abundance. J. Mass Spectrom. 31, 255–262.
Field, C.J., Blewett, H.H., Proctor, S., Vine, D., 2009. Human health benefits of vaccenic acid. Applied physiology, nutrition, and metabolism = Physiologie appliquee, nutrition et metabolisme 34, 979–991.
Francis, S.A., Kelly, J.M., McCormack, J., Rogers, R.A., Lai, J., Schneeberger, E.E., Lynch, R.D., 1999. Rapid reduction of MDCK cell cholesterol by methyl-beta-cyclodextrin alters steady state transepithelial electrical resistance. Eur. J. Cell Biol. 78, 473–484.
Gomez, J.D., Wall, M.L., Rahim, M., Kambhampati, S., Evans, B.S., Allen, D.K., Antoniewicz, M.R., Young, J.D., 2023. Program for integration and rapid analysis of mass isotopomer distributions (PIRAMID). Bioinformatics 39.
Gonzalez, J.E., Antoniewicz, M.R., 2017. Tracing metabolism from lignocellulosic biomass and gaseous substrates to products with stable-isotopes. Curr. Opin. Biotechnol. 43, 86–95.
Gonzalez, J.E., Naik, H.M., Oates, E.H., Dhara, V.G., McConnell, B.O., Kumar, S., Betenbaugh, M.J., Antoniewicz, M.R., 2024. Comprehensive stable-isotope tracing of glucose and amino acids identifies metabolic by-products and their sources in CHO cell culture. Proc. Natl. Acad. Sci. USA. 121, e2403033121.
Harayama, T., Antonny, B., 2023. Beyond fluidity: the role of lipid unsaturation in membrane function. Cold Spring Harbor Perspect. Biol. 15.
Ladiwala, P., Cai, X., Naik, H.M., Aliyu, L., Schilling, M., Antoniewicz, M.R., Betenbaugh, M.J., 2024. Ala-Cys-Cys-Ala dipeptide dimer alleviates problematic cysteine and cystine levels in media formulations and enhances CHO cell growth and metabolism. Metab. Eng. 85, 105–115.
Ladiwala, P., Dhara, V.G., Jenkins, J., Kuang, B., Hoang, D., Yoon, S., Betenbaugh, M.J., 2023. Addressing amino acid-derived inhibitory metabolites and enhancing CHO cell culture performance through DOE-guided media modifications. Biotechnol. Bioeng. 120, 2542–2558.
Li, Z.M., Fan, Z.L., Wang, X.Y., Wang, T.Y., 2022. Factors affecting the expression of recombinant protein and improvement strategies in Chinese hamster ovary cells. Front. Bioeng. Biotechnol. 10, 880155.
Long, C.P., Antoniewicz, M.R., 2019. High-resolution (13)C metabolic flux analysis. Nat. Protoc. 14, 2856–2877.
Ma, Y., Temkin, S.M., Hawkridge, A.M., Guo, C., Wang, W., Wang, X.Y., Fang, X., 2018. Fatty acid oxidation: an emerging facet of metabolic transformation in cancer. Cancer Lett. 435, 92–100.
Mahammad, S., Parmryd, I., 2015. Cholesterol depletion using methyl-beta-cyclodextrin. Methods Mol. Biol. 1232, 91–102.
Maulucci, G., Cohen, O., Daniel, B., Sansone, A., Petropoulou, P.I., Filou, S., Spyridonidis, A., Pani, G., De Spirito, M., Chatgilialoglu, C., Ferreri, C., Kypreos, K. E., Sasson, S., 2016. Fatty acid-related modulations of membrane fluidity in cells: detection and implications. Free Radic. Res. 50, S40–S50.
McLafferty, F.W., 1959. Mass spectrometric analysis. Molecular rearrangements. Anal. Chem. 31, 82–87.
Naik, H.M., Cai, X., Ladiwala, P., Reddy, J.V., Betenbaugh, M.J., Antoniewicz, M.R., 2024. Elucidating uptake and metabolic fate of dipeptides in CHO cell cultures using (13)C labeling experiments and kinetic modeling. Metab. Eng. 83, 12–23.
Naik, H.M., Kumar, S., Reddy, J.V., Gonzalez, J.E., McConnell, B.O., Dhara, V.G., Wang, T., Yu, M., Antoniewicz, M.R., Betenbaugh, M.J., 2023. Chemical inhibitors of hexokinase-2 enzyme reduce lactate accumulation, alter glycosylation processing, and produce altered glycoforms in CHO cell cultures. Biotechnol. Bioeng. 120, 2559–2577.
Oates, E.H., Antoniewicz, M.R., 2022. Coordinated reprogramming of metabolism and cell function in adipocytes from proliferation to differentiation. Metab. Eng. 69, 221–230.
Oates, E.H., Antoniewicz, M.R., 2023. (13)C-Metabolic flux analysis of 3T3-L1 adipocytes illuminates its core metabolism under hypoxia. Metab. Eng. 76, 158–166.
Ramirez, O.T., Mutharasan, R., 1990. The role of the plasma membrane fluidity on the shear sensitivity of hybridomas grown under hydrodynamic stress. Biotechnol. Bioeng. 36, 911–920.
Ritacco, F.V., Wu, Y., Khetan, A., 2018. Cell culture media for recombinant protein expression in Chinese hamster ovary (CHO) cells: history, key components, and optimization strategies. Biotechnol. Prog. 34, 1407–1426.
Rivier, A.S., Castillon, G.A., Michon, L., Fukasawa, M., Romanova-Michaelides, M., Jaensch, N., Hanada, K., Watanabe, R., 2010. Exit of GPI-anchored proteins from the ER differs in yeast and mammalian cells. Traffic 11, 1017–1033.
Settles, E.I., Loftus, A.F., McKeown, A.N., Parthasarathy, R., 2010. The vesicle trafficking protein Sar1 lowers lipid membrane rigidity. Biophys. J. 99, 1539–1545.
Stella, V.J., Rajewski, R.A., 1997. Cyclodextrins: their future in drug formulation and delivery. Pharm. Res. 14, 556–567.
Subbaiah, P.V., Gould, I.G., Lal, S., Aizezi, B., 2011. Incorporation profiles of conjugated linoleic acid isomers in cell membranes and their positional distribution in phospholipids. Biochim. Biophys. Acta 1811, 17–24.
Szeliova, D., Ruckerbauer, D.E., Galleguillos, S.N., Petersen, L.B., Natter, K., Hanscho, M., Troyer, C., Causon, T., Schoeny, H., Christensen, H.B., Lee, D.Y., Lewis, N.E., Koellensperger, G., Hann, S., Nielsen, L.K., Borth, N., Zanghellini, J., 2020. What CHO is made of: variations in the biomass composition of Chinese hamster ovary cell lines. Metab. Eng. 61, 288–300.
van der Valk, J., Brunner, D., De Smet, K., Fex Svenningsen, A., Honegger, P., Knudsen, L. E., Lindl, T., Noraberg, J., Price, A., Scarino, M.L., Gstraunthaler, G., 2010. Optimization of chemically defined cell culture media–replacing fetal bovine serum in mammalian in vitro methods. Toxicol. Vitro 24, 1053–1063.
Whelan, J., Fritsche, K., 2013. Linoleic acid. Adv. Nutr. 4, 311–312.
Zidovetzki, R., Levitan, I., 2007. Use of cyclodextrins to manipulate plasma membrane cholesterol content: evidence, misconceptions and control strategies. Biochim. Biophys. Acta 1768, 1311–1324.