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Journal of Chromatography B, 810 (2004) 119–127
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Micro-quantitation of lipids in serum-free cell culture media: a critical aspect is the minimization of interference from medium components and chemical reagents

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Received 26 February 2004; accepted 19 July 2004
Available online 26 August 2004

Abstract

Lipids (fatty acids) at a concentration range of 10–100 μg/L are essential components included in most serum-free cell culture medium formulations. A gas chromatography/mass spectrometry (GC/MS) method for the micro-quantitation of lipids, determined as fatty acid methyl esters (FAMEs), in complex serum-free cell culture media was developed. The interference of derivatizing reagents, extraction solvents and medium additives in the micro-quantitation of lipids was also examined. The results show that the concentration of fatty acids such as palmitic and stearic acids detected in derivatizing reagents or extraction solvents was in the range of 10–230 μg/L. Tween-80, a surfactant and medium additive, produced nearly 20 FAMEs alone when methylated using a derivatizing agent. Moreover, the surfactant Pluronic F-68, a medium additive, interfered in the FAME recovery. Procedures, which include use of low volumetric ratio of reagent to medium and precipitation of the above surfactants, were developed to minimize background FAMEs to levels which do not significantly affect the quantitation of medium lipids and to diminish the interference caused by Pluronic F-68. Fatty acid concentrations in several complex serum-free culture media were quantitated by this method and were very close to the values indicated in their formulations.

Keywords: Lipids; Cell culture medium

1. Introduction

Lipids are very important constituents of cells. They are the main components of membranes and provide permeability barriers that are essential for cell survival and function. Most serum-free cell culture medium formulations include essential fatty acids to replace the growth-promoting properties of the lipid components of serum [1]. Fatty acids are one type of lipids; however, the terms of lipids and fatty acids will be employed without distinction throughout this text. The concentration of the essential fatty acids in most classic mammalian cell serum-free media [2] and currently available proprietary serum-free media is usually in the range of 10–100 μg/L (ppb). The lipid concentration in insect cell serum-free media however is much higher, in the range of 1000 μg/L [3]. Supplements of fatty acids in serum-free cell culture media have been recognized as essential to stimulate cell growth [4,5] and to improve the robustness of cells in agitated cultures [6].

Accurate and precise quantitation of lipids in cell culture media is important for media quality control and in metabolic

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doi:10.1016/j.jchromb.2004.07.020

study of lipids in cell culture processes [7]. Gas chromatography coupled with mass spectrometry (GC/MS) has been extensively used for the quantitation of lipids through fatty acid analysis [8]. In this quantitation process, fatty acids in samples are converted into fatty acid methyl esters (FAMEs). The derivatizing methods for the preparation of FAMEs have been reviewed by Blau and Darbre [9]. However, most of these methods have been applied to standards or samples containing milligrams of lipid-dominant components. The potential interference from impurities in derivatizing reagents and solvents, which might be critical in the lipid micro-quantitation, was not considered in these methods. In addition, some components, such as the non-ionic surfactants Tween-80 (or known as polyxyethylene sorbitan monooleate, Merck Index 7742) and Pluronic F-68 (PF-68, also known as poloxamers or polyethylenepolypropylene glycols, Merck Index 7722), in complex cell culture media could potentially interfere in the lipid quantitation. For example, fatty acid esters will be generated once Tween-80 is methylated using a derivatizing agent, impairing the quantitation of "real" lipids in the media. As well, the emulsification properties of PF-68 interfere in lipid extraction and recovery. To our knowledge, there are no reports that describe quantitative analysis of lipids at micro levels (<100 μg/L) in complex culture media.

The objectives of the present study were to examine the interference of media components and chemical reagents in the micro-quantitation of lipids, and to eliminate or at least minimize their interferences in order to develop an accurate method for micro-quantitation of lipids in serum-free cell culture media.

2. Experimental

2.1. Reagents and solutions

BF_3–methanol solutions, having concentrations of BF_3 of 10 and 14%, were purchased from Sigma (St. Louis, MO), Supelco (Bellefront, PA) and Fluka Chemie GmbH (Buchs, Germany), respectively. SAP-ESTER Kit (consisting of reagent A; 0.5N methanolic NaOH and reagent B; 14% BF_3/methanol) was purchased from Alltech Associates Inc. (Deerfield, IL). All BF_3 reagents were stored at 4 °C and used within 2 months upon receipt. Hexane and ethyl acetate (OmniSolv grade), methanol and dichloromethane (OmniSolv HR-GC grade), and chloroform (GR ACS grade) were all obtained from EM Science (Merck KgaA, Darmstadt, Germany). Acetyl chloride (99+%) and nonadecanoic acid (99%) were purchased from Aldrich (Milwaukee, WI). The HCl–methanol reagent was prepared by slowly adding 2 ml of acetyl chloride to 10 ml of methanol at −20 °C. Ammonium thiocyanate and cobalt nitrate were purchased from Sigma and are ACS reagents. The cobalt thiocyanate solution was prepared by dissolving 3 g of cobalt nitrate and 20 g of ammonium thiocyanate in 100 ml of Milli-Q water. Supelco™ 37 component FAME mix (Catalog No. 47885-U), a chemically defined lipid mixture (Catalog No. L 0288), Tween-80, Pluronic F-68 and sodium chloride were purchased from Sigma, and the last three chemicals are cell culture grade. New glasswares were used in the experiments except for micro-reaction vessel and separatory funnel. The latter were carefully cleaned, rinsed twice with CH_2Cl_2 and then with methanol, and dedicated to this experiment only. Rubber glove was always worn during the sample preparation.

The mammalian cell culture media included DME/F-12 (1:1 mixture), Medium 199 (both from Sigma), DM- (a customized medium from BioWhittaker, Walksersville, MD), 293F, LC-SFM (both from Invitrogen, Burlington, Ont.) and NSFM 13 (a NRC proprietary medium). The insect cell culture media used included Sf-900 II (Invitrogen), Ex-Cell 405 (JRH Biosciences, Lenexa, KS) and IPL 41 (Sigma). A complete IPL 41 medium was prepared by supplementing the IPL 41 basal medium with a lipid mixture according to Inlow et al. [3].

2.2. Examination of background fatty acid signals due to derivatizing reagents

Effects on the background signal caused by varying the amount of BF_3 agent (10% BF_3–methanol, Fluka), reaction temperature and length of reaction were examined by a set of experiments carried out in 5 ml micro-reaction vessels (Supelco). Two levels were chosen for each parameter: 0.25 and 2.5 ml of 10% BF_3–methanol solution, 60 and 90 °C, and 10 and 60 min. Following the reaction, the samples were cooled to room temperature and then mixed with 0.5 ml of NaCl saturated water before extraction with 2 × 0.5 ml of hexane. The top organic layers were pooled in 2 ml glass vials and hexane was then evaporated under vacuum. The dried extract was dissolved in 100 μl of ethyl acetate for subsequent GC/MS analysis. The background of other BF_3 agents was examined by using 2.5 ml BF_3 agent and heating at 60 °C for 10 min.

A similar approach was used to assess the fatty acid background of the HCl–methanol derivatizing reagent. The two levels chosen for each parameter were 0.20 and 2.0 ml of the reagent, 70 and 90 °C, and 30 and 120 min.

2.3. Examination of background fatty acid signals due to solvents and other chemical reagents

Ten milliter of CH_2Cl_2 or $CHCl_3$ was evaporated under a nitrogen stream. The residues were derivatized with 0.20 ml of the HCl–methanol reagent at 70 °C for 30 min. The background sample for acetyl chloride was prepared by extracting 0.20 ml acetyl chloride with 2 × 0.5 ml CH_2Cl_2, evaporating CH_2Cl_2, and derivatizing the residue. The background of methanol was prepared by evaporating 2 ml of methanol and derivatizing the residue. A sample representing a pooled background from chemicals (1 ml of hexane, 100 μl of ethyl acetate and 0.5 ml of NaCl saturated water) was also prepared for subsequent GC/MS analysis of suspected FAMEs.

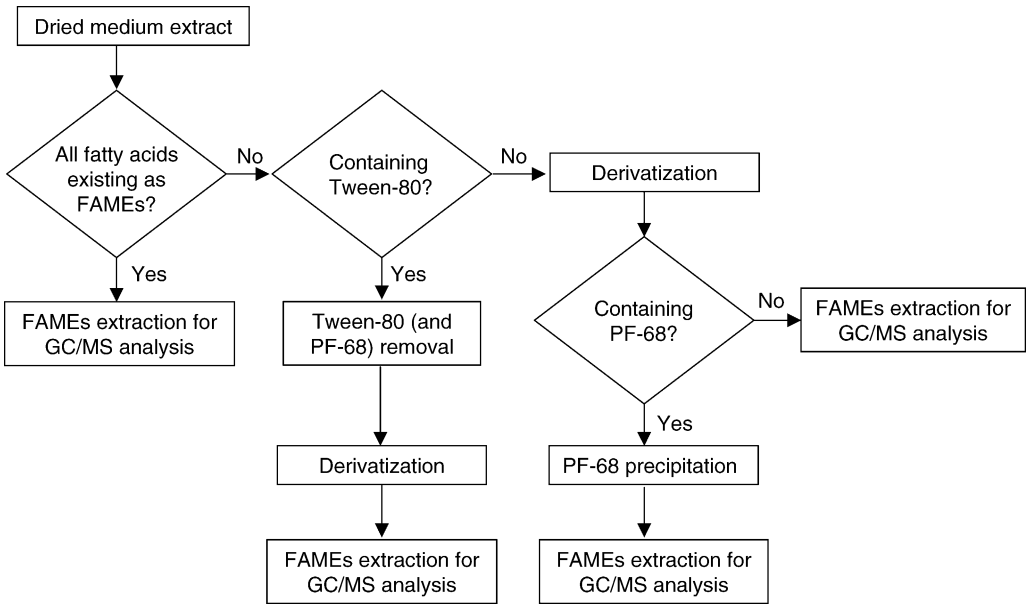

Fig. 1. Scheme of the preparation of FAME sample from medium extract for GC/MS analysis.

2.4. Preparation of FAMEs from serum-free cell culture media

Fifty millilitres of mammalian or insect cell culture medium was added into a 125 ml separatory funnel and treated with 6 N HCl solution to convert any fatty acid salts to free acids. The fatty acids were extracted with CH_2Cl_2 (2 × 5 ml) by vigorously shaking the mixture for about 1 min. The organic layer was collected in a 5 ml micro reaction vessel, and evaporated to dryness under a gentle stream of nitrogen gas. The preparation of FAME samples from the dried medium extract is schematically illustrated in Fig. 1, showing that the procedure was dependent on the medium composition. Following this strategy, the sample derivatization was carried out by reacting with 0.25 ml of 14% BF_3–methanol solution (Sigma) at 60 °C for 10 min, and the FAME extraction was performed by repeatedly extraction with hexane (5 × 0.5 ml). The procedures for Tween-80 removal and PF-68 precipitation are detailed below.

2.5. Examination of background from Tween-80

A medium extract prepared from Medium 199 or IPL 41 (both containing Tween-80 in their formulations) was dissolved in 0.45 ml of methanol, and then mixed with 0.15 ml of the cobalt thiocyanate solution to precipitate Tween-80 (and PF-68 if any). Separation of the lipid fraction from the Tween-80 precipitate was preformed by multiple extraction of the mixture with 5 × 0.5 ml of hexane. The recovered lipids (fatty acids and/or FAMEs) in the hexane layer were then derivatized. For comparison, parallel sets of medium extract from Medium 199 or IPL 41 were derivatized without Tween-80 precipitation.

2.6. Effect of PF-68 presence on FAME extraction and recovery

A set of medium extracts, each prepared from 50 ml of DME/F12 medium spiked with 100 μl of the FAME mixture (containing 37 FAMEs) and 50 μl of 25 mg/L nonadecanoic acid methanol solution, were derivatized with BF_3–methanol. Some derivatized samples were spiked with 200 μl of 200 g/L PF-68 methanol solution before the extraction with 0.5 ml hexane.

Phase separation for samples containing PF-68 was performed by either centrifugation at 2000 × *g* for 5 min or by precipitation. The later was achieved by mixing the sample with 0.70 ml of cobalt thiocyanate solution. After the phase separation, the top hexane layer was transferred to a 2 ml glass tube. Some samples were extracted further with 0.5 ml hexane while others with 4 × 0.5 ml hexane. No additional cobalt thiocyanate was added after the first extraction.

2.7. GC/MS analysis of FAMEs

The analysis and quantitation of FAMEs were carried out using an HP6890 gas chromatograph coupled with a 5973 mass-selective detector (Agilent Technologies, Palo Alto, CA). The GC column was a SP-2330 (Supelco, Oakville, Ont.) capillary column (30 m × 0.20 mm i.d., 0.20 μm film thickness). Helium carrier gas flow-rate was 1 ml/min. One microliter of sample was injected in splitless mode. The column temperature was programmed from 100 to 250 °C at a rate of 10 °C/min. The temperature of both injector and detector was 280 °C. Full scan electron ionization mass spectra were obtained over a range of 50–500 u. Data collection and integration were performed with HP Chem Station software. The identification of FAMEs was performed by library search

Table 1
Fatty acid background detected in derivatizing reagents

Derivatizing reagent	Fatty acid concentration (μg/L)[a]					
	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2
10% BF_3–methanol (Fluka)	–	118 ± 3[c]	–	205 ± 21	–	–
14% BF_3–methanol (Sigma)	–	104	–	110	12	–
10% BF_3–methanol (Supelco)	–	60	–	138	8	–
SAP-ESTER kit (Alltech)	58 ± 5	179 ± 12	47 ± 9	96 ± 9	82 ± 9	18 ± 8
HCl–methanol[b]	–	56 ± 20	–	229 ± 12	–	–
Acetyl chloride (Aldrich)	–	450	–	775	–	–

Note: methylation with BF_3–methanol was conducted at 60 °C and for 10 min, while the methylation with HCl–methanol was at 70 °C and for 30 min.
[a] Fatty acids: C14:0 (myristic acid), C16:0 (palmitic acid), C16:1 (palmitoleic acid), C18:0 (stearic acid), C18:1 (oleic acid) and C18:2 (linoleic acid).
[b] HCl–methanol was prepared by adding 2 ml of acetyl chloride to 10 ml of methanol at −20 °C.
[c] The standard deviation was from duplicate tests.

and comparison with standard compounds when applicable. The concentration of FAMEs in the sample was either quantitated by comparison with an external standard prepared from the Supelco™ 37 component FAME mix or using nonadecanoic acid (C19:0) as internal standard.

3. Results and discussion

3.1. Analysis of background signals

3.1.1. Derivatizing agents

Table 1 shows that fatty acids such as palmitic acid (C16:0), stearic acid (C18:0) and oleic acid (C18:1) were detected in the background generated from different BF_3–methanol reagents, such as those from Fluka, Sigma and Supelco. However, pronounced concentrations of other fatty acids such as palmitoleic acid, oleic acid and linoleic acid were also detected in the background generated from some lots of the SAP-ESTER Kit (Alltech). FAMEs at lower concentrations were also detected in the samples prepared from BF_3 reagents without the heating step (60 °C at 10 min), indicating that some of the fatty acid background in the BF_3 reagents were present as FAMEs. No artifacts, such as the formation of methoxylated fatty acids, were detected, although BF_3 has been reported to produce this type of compound [10].

Results from the examination of 10% BF_3–methanol reagent (Fluka) under different experimental conditions show that the background fatty acid concentrations increased with increasing amounts of BF_3 reagent used in the derivatizing process, while the reaction temperature and duration did not contribute significantly (data not shown).

We also detected fatty acids (C16:0 and C18:0, Table 1) and many other non-fatty acid components in the background from the HCl–methanol reagent (results not shown). Also, it was found that impurities in the acetyl chloride were a main source of the background. The effect of experimental conditions on the fatty acid background (data not shown) indicates that, in general, the background of C16:0 and C18:0 increased when the volume of the HCl–methanol reagent was increased. Furthermore, the background was slightly higher when the sample was heated to elevated temperatures and/or extended duration, which may be due to enhanced conversion of fatty acids to FAMEs under more vigorous conditions.

In general, we found that the concentration of individual fatty acid (typically C16:0 and C18:0 detected as FAMEs) in derivatizing agent could be as high as 230 μg/L. The amount of these fatty acids added by the derivatizing agent during the sample preparation process is proportional to the volume of reagent, and their influence on the medium lipid quantitation varies inversely to the employed medium volume. In other words, the influence of interfering fatty acids on the medium lipid quantitation is directly related to the volumetric ratio of derivatizing agent to medium, or called “dilution factor”. For example, when 2.5 ml of reagent (typically suggested by manufacturer) is used to derivatize a medium extract prepared from 5 ml of medium (ratio = 0.5), the concentration of individual interfering compound (such as C18:0) added by the reagent could be as high as 115 μg/L when reported as the result of medium lipid quantitation. This concentration is in the same concentration range of lipid present in mammalian cell culture media; therefore, background concentrations from the derivatizing agents could significantly affect the quantitation of lipid concentrations in the medium. However, the interference of C18:0 can be reduced to less than 2 μg/L if the medium volume is increased to 50 ml and the volume of derivatizing reagent is reduced to 0.25 ml (ratio = 0.005). This ratio (0.005) was used in the medium sample preparation.

BF_3 and HCl–methanol reagents are two of the most popular reagents for esterification [9]. Table 1 shows that the fatty acid backgrounds from BF_3–methanol or HCl–methanol reagents were in the same range. However, BF_3–methanol reagent provided rapid methylation and produced less non-fatty acid components that also contribute to the background; therefore, it was chosen as the derivatizing agent for subsequent samples prepared from cell culture media.

3.1.2. Organic solvents

Table 2 shows that C16:0 and C18:0 fatty acids were common contaminants in most of the solvents used. Depending on the reagent grade and supplier of solvent, the background

Table 2
Fatty acid background detected in chemical reagents

Reagent	Source	Fatty acid concentration (μg/L)[a]		
		C14:0	C16:0	C18:0
Dichloromethane	EM Science, OmniSolv HR-GC grade	–	6	12
Dichloromethane	Aldrich, pesticide residue analysis grade	6	88	192
Chloroform	EM Science, GR ACS	3	36	63
Methanol	EM Science, OmniSolv HR-GC grade	–	30	50

[a] Abbreviation as in Table 1.

fatty acid concentration (such as C18:0) varied from 12 to 192 μg/L. Also like the derivatizing agent, the influence of fatty acid background from solvents on the medium lipid quantitation was also dependent on the volumetric ratio of solvent to medium. The fatty acid (such as C18:0) contributed by the three different chlorinated solvents to the medium lipid quantitation would vary from 3 to 40 μg/L when 10 ml of each solvent would be used to extract 50 ml of medium (the solvent/medium ratio was 0.2 and this ratio was used in the medium sample preparation). No fatty acid methyl esters were detected in the sample representing a pooled background from chemicals (1 ml of hexane, 100 μl of ethyl acetate and 0.5 ml of NaCl saturated water).

The presence of small amounts of fatty acids in organic solvents may be due to contamination during the manufacturing and packaging processes in the form of leachates from plasticizers used in the container caps. Plasticizers commonly consist of fatty acids esterified with ethyl-, butyl-, and longer-chain alcohols [11]. Fatty acid contamination from plastic packaging has been frequently reported by laboratories interested in lipid quantitation [12]. Our experimental results indicate that most organic solvents were common sources of trace fatty acid contaminants, and solvents with the highest purity should be used in micro lipid quantitation procedures.

3.2. Effect of medium additives on quantitation of FAMEs

3.2.1. Tween-80

Tween-80 is a component in the lipid supplement for cell culture, and is included in formulations of some media (Sigma catalogue, 2002). Oleic acid methyl ester is generated when the medium extract containing Tween-80 is methylated by derivatizing agents. To investigate the influence of Tween-80 in the medium on FAME quantitation, we chose a chemically defined classical medium, Medium 199 which contains 20 mg/L of Tween-80. According to the medium formulation provided by the manufacturer, Tween-80 is the only fatty acid producing component in this medium (Sigma catalogue, 2002). Fig. 2 shows the GC/MS total ion chromatogram of the sample prepared from Medium 199, and also indicates that the relative retention time of each peak to the internal standard ranges from 0.61 to 1.1. The identification and quantitation of the peaks presented in Table 3 shows that, in addition to 1415 μg/L of oleic acid methyl ester, other fatty acids were also detected, and their concentrations ranged from 3 to 80 μg/L or in the range of 0.2–5.6% of the oleic acid concentration. The concentrations of these fatty acids are significant when compared to the lipid concentrations (from 10 to 100 μg/L) found in mammalian cell culture media. These fatty acids generated from Tween-80 will no doubt cause a severe positive bias in lipid quantitation in media containing Tween-80. Many fatty acids detected in Medium 199 were probably from impurities originally present in the oleic acid that was used to produce Tween-80.

The influence of Tween-80 in cell culture media on the fatty acid profile was further examined by analyzing fatty acid compositions in the DME/F12 medium spiked with 5 mg/L of Tween-80 (Sigma) and in the chemically defined lipid (containing 2200 mg/L of Tween-80) from Sigma (Catalogue No. L0288). The detected fatty acids were compared to those listed in their respective formulations. Experimental results (data not shown) revealed that additional fatty acids were detected in the DME/F12 medium spiked with Tween-80 and in the chemically defined lipid.

To minimize the interference of Tween-80 in the medium lipid quantitation, Tween-80 in the medium extract was precipitated by forming a complex with cobalt thiocyanate [13]. The principle of precipitation is based on that the oxygen groups in the polyether chain of non-ionic detergents (such as Tween-80 or PF-68) complex with an inorganic cation (ammonium or cobalt ion) to form oxonium ion which reacts with a relatively large anion (thiocyanate) to produce a salt. This salt precipitates out from aqueous solution and is then separated from the lipid fraction. Table 3 (column 4) shows that the fatty acid background from Tween-80 was dramatically reduced when Tween-80 was removed before the

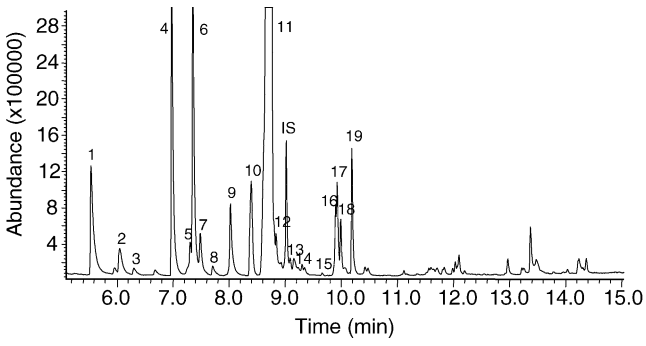

Fig. 2. FAMEs generated from Tween-80 present in Medium 199. Identification and quantitation of the peaks were listed in Table 3.

Table 3
Effect of Tween-80 removal (TWRV) on the fatty acids and their concentrations detected in the Medium 199 (M199) and 293F

Peak #	Fatty acids	Fatty acid concentrations (μg/L)		
		M199 without TWRV	M199 with TWRV	50% M199 + 50% 293F with TWRV
1	Myristic acid (C14:0)	39.9 ± 2.7	6.1 ± 1.5	4.7 ± 2.1
2	Myristoleic acid (C14:1)	11.5 ± 0.8		
3	Pentadecanoic acid (C15:0)	2.3 ± 0.3		
4	Palmitic acid (C16:0)	22.9 ± 0.9	12.2 ± 1.1	12.9 ± 4.0
5	C16:1 isomer[a]	7.2 ± 0.2		
6	Palmitoleic acid (C16:1)	82.6 ± 3.0	5.1 ± 0.9	2.5 ± 0.7
7	C16:1 isomer[a]	14.3 ± 2.4		
8	Heptadecanoic acid (C17:0)	2.9 ± 0.7		
9	Heptadecenoic acid (C17:1)	23.1 ± 1.8		
10	Stearic acid (C18:0)	11.3 ± 1.6	9.3 ± 2.3	11.2 ± 5.9
11	Oleic acid (C18:1)	1572 ± 222	142.3 ± 8.2	77.9 ± 14.7
12	C18:1 isomer[a]	5.2 ± 4.6		
13	Linolelaidic acid (C18:2)	3.2 ± 1.5		
14	Linoleic acid (C18:2)	4.2 ± 0.7		30.4 ± 4.5
15	Arachidic acid (C20:0)	0.3 ± 0.2		
16	C18:2 isomer[a]	10.5 ± 1.2		
17	Eicosenoic acid (C20:1)	14.2 ± 4.7		
18	C18:2 isomer[a]	10.2 ± 0.6		
19	C18:2 isomer[a]	28.7 ± 3.0		

[a] The concentration of these fatty acids was calculated by using the same response factor of the known isomer.

derivatization of the medium extract. As a result of the precipitation procedure there was a 91% reduction in the oleic acid concentration, indicating that most Tween-80 was removed. Small amounts of other fatty acids were more likely from the derivatizing agent and solvent as previously mentioned. To verify whether the Tween-80 removal would affect the recovery of fatty acids and accuracy of medium lipid quantitation, the lipid concentration in a medium mixture (50% medium 199 and 50% 293F) was analyzed by applying the Tween-80 removal process. The experimental data shows that 60% of linoleic acid was recovered, but the concentration of linoleic acid (30.4 ± 4.5 μg/L, Table 3) detected by an internal standard method was close to 30 μg/L, or 50% of the 60 μg/L linoleic acid included in the 293F medium formulation. Results from additional experiments indicate the recovery of fatty acids was dramatically improved to higher than 90% if the individual fatty acid in media was at higher concentrations such as 200 μg/L. This result suggests that the process of Tween-80 removal may affect the recovery of fatty acids, but would not significantly affect the accuracy of medium lipid quantitation if an appropriate quantitation method is used.

The interference caused by the presence of Tween-80 (25 mg/L) in the lipid quantitation of IPL-41 medium which has complex and high FAME concentrations is presented in Table 4. Since the lipid mixture added to the IPL-41 medium was prepared from cod liver oil FAMEs, it was possible to efficiently extract them from the medium using CH_2Cl_2. Therefore, the result obtained by the direct extraction (Table 4, column 2) represented the real concentration of FAMEs in the IPL 41 medium and was used as a control. The data in Table 4 reveal that the concentration of some fatty acids in the sample prepared without Tween-80 removal was much higher than that in the control, while the concentration of other fatty acids was very close to those found in the control. With the removal of Tween-80, the concentration of all fatty acids detected was close to the control. These results also indicate that, due to the high lipid concentration present in the insect cell culture medium, the fatty acids (even oleic acid) generated from the residual Tween-80 after its removal did not dramatically change the fatty acid concentrations detected in the medium. Data from the analysis of other media (not shown) also indicate that, except for oleic acid, other fatty acids from Tween-80 were eliminated by the Tween-80 removal procedure. Based on the above results, we can conclude that it is feasible to accurately quantitate FAMEs and fatty acids (except for oleic acids) in the serum-free media containing Tween-80 after precipitation with cobalt thiocyanate.

3.2.2. Influence of PF-68 on the extraction and recovery of FAMEs

PF-68, another synthetic non-ionic surfactant, is usually added in serum-free media at concentrations in the range of 0.3–1 g/L to protect the cells from shear stress, typically in sparged bioreactors. During the extraction of lipids from the medium using CH_2Cl_2, up to 90% of the PF-68 was also extracted. The presence of a large amount of the extracted PF-68 in the BF_3–methanol derivatized sample caused a serious emulsification problem during the FAME extraction with hexane, making the separation of the organic and aqueous phases very difficult and causing poor recovery of the organic phase, which includes the FAMEs. To evaluate the influence of the presence of PF-68 on the recovery of different FAMEs, samples were prepared from FAME standard supplemented

Table 4
Effect of Tween-80 removal (TWRV) on the fatty acid concentrations detected in IPL-41 medium[a]

Fatty acids	Fatty acids (μg/L) detected in samples prepared under different procedures[a]		
	P1: direct extraction	P2: w/o TWRV	P3: with TWRV
Myristic acid (C14:0)	363	513	305
Pentadecanoic acid (C15:0)	23	36	26
Palmitic acid (C16:0)	1076	1432	1060
Palmitoleic acid (C16:1)	792	1096	724
Heptadecanoic acid (C17:0)	95	124	98
cis-10-Heptadecenoic acid (C17:1)	51	137	52
Stearic acid (C18:0)	250	380	276
Elaidic acid (C18:1n9t)	57	256	65
Oleic acid (C18:1n9c)	1709	6268	2077
C18:1 isomer[b]	589	1007	572
Linoleic acid (C18:2n6c)	254	297	262
Arachidic acid (C20:0)	12	12	14
Linolenic acid (C18:3n3)	103	106	105
cis-11-Eicosenoic acid (C20:1)	1194	1167	1151
C20:1 isomer[b]	195	556	190
C18:3 isomer[b]	31	30	31
cis-11,14-Eicosadienoic acid (C20:2)	6	9	7
cis-11,14,17-Eicosatrienoic acid (C20:3n3)	59	63	62
Arachidonic acid (C20:4n6) + *cis*-11, 14, 17-eicosatrienoic acid (C20:3)	980	896	1034
Erucic acid (C22:1n9)	74	79	83
C20:4 isomer[b]	83	85	86
cis-5,8,11,14,17-Eicosapentaenoic acid (C20:5)	591	613	614
Nervonic acid (C24:1)	71	74	67
cis-4,7,10,13,16,19-Docosahexaenoic acid (C22:6n3)	1343	1481	1449

[a] The fatty acids in IPL-41 medium were added as methyl esters (FAMEs). P1: a direct extraction of this medium, without methylation of medium extract, original fatty acid composition. P2: methylation without Tween-80 removal. P3: methylation with Tween-80 removal.
[b] Noted as in Table 3.

DME/F12 medium, which does not contain PF-68 in its formulation. Some derivatized samples were spiked with 40 mg of PF-68 (equivalent to the amount extracted from 50 ml of medium containing PF-68 at 1 g/L) to mimic the effect of PF-68 on the FAME extraction, and to compare recoveries in the presence and absence of PF-68. Experimental data (not shown) indicate that the recovery of 28 individual FAMEs (from C14 to C24 with different saturations) in the controls without PF-68 was higher than 80%. In contrast, FAME recovery in the sample in presence of PF-68 and extracted twice with hexane was very low, ranging from 2 to 22%. In fact, the phase separation was very poor even after the sample was centrifuged at 2000 × *g* for 5 min, and only a small portion of the hexane extract was recovered. By increasing the number of hexane extractions to five, the FAME recovery was improved, ranging from 20 to 86%. However, individual FAME recovery, especially saturated FAMEs with long carbon chains, was still low.

With the addition of 0.7 ml of cobalt thiocyanate solution to the derivatized sample, the PF-68 was immediately precipitated, resulting in a clear separation of the organic and aqueous phases. The experimental data (not shown) indicate that, with the precipitation of PF-68, the recovery of FAMEs was dramatically improved in the sample extracted twice with hexane. The recovery of most FAMEs was higher than 80%. When the sample was extracted five times with hexane, the recovery of FAMEs was further improved, reaching more than 85% for most FAMEs except for C22:0, C23:0, C24:0 and C24:1. The recoveries of these four FAMEs ranged from 50 to 71%. Similar effects of the presence of PF-68 on the recovery of FAMEs were observed when media containing 1 g/L PF-68 was used for the sample preparation (data not shown).

3.3. Quantitation of lipid in serum-free cell culture media

3.3.1. Mammalian cell culture medium

Fatty acid concentrations in 5 different media with various concentration of PF-68 were quantified using C19:0 as an internal standard after precipitating PF-68. Table 5 (column 7) shows that linoleic acid was the sole fatty acid included in the formulation of most mammalian cell culture media and its concentration quantitated by our process was very close to its formulation data. The data also show that the presence of PF-68 at various concentrations (0–1 g/L) as shown in column 8 did not affect the accuracy of lipid quantitation. Some other fatty acids, such as C16:0 and C18:0, not included in the medium formulation, were also detected but at much lower concentrations. The presence of these fatty acids was more likely a result of the contribution of the extraction solvent and the derivatizing agent as mentioned previously.

Table 5
Fatty acids detected in serum-free cell culture media with defined fatty acid and PF-68 concentrations in their formulations

Medium	Fatty acids[a] detected (μg/L)					Fatty acid (μg/L) and PF-68 (g/L) included in the medium formulation	
	C16:0	C16:1	C18:0	C18:1	C18:2	C18:2	PF-68
DME/F12	7.3 ± 0.7	0.5 ± 0.7	9.5 ± 2.4	1.4 ± 0.2	40.9 ± 1.9	42	0
293F	3.1 ± 0.1	–	4.6 ± 0.3	–	63.4 ± 1.4	60	0.3
NSFM 13	7.1	–	18.2	–	38.7	40	0.87
DM-	6.3	–	11.6	–	0	0	1.0
LC-SFM	6.1 ± 0.9	–	8.8 ± 2.1	–	37.7 ± 4.5	35	1.0

[a] Fatty acid abbreviations as in Table 1.

3.3.2. Insect cell culture medium

Fatty acid concentration in IPL-41 medium is presented in Table 4. The fatty acid concentration in Sf-900 II was also examined and the fatty acid profile was similar to that found in the IPL 41 medium except for variations in some fatty acid concentration, indicating cod liver oil fatty acids were more likely added in this medium as the source of lipids. While the fatty acid analysis of the medium EX-CELL 405 reveals that linoleic acid (C18:2) and linolenic acid (C18:3), at a respective concentration of 500 ppb each, were added as the sources of lipids, although other fatty acids (at low concentrations) probably from the derivatizing agent, solvent and Tween-80 were also detected. The above data indicate that the lipid concentrations in serum-free insect cell culture media were much higher than that found in mammalian cell culture media. These results are consistent with the lipid concentrations usually reported for insect cell culture media [3].

The results of lipid quantitation in serum-free cell culture media indicate that accurate micro lipid quantitation can be achieved through the application of processes developed for serum-free media with different compositions. The strategy developed for sample preparations is schematically illustrated in Fig. 1 and can be briefly summarized as follows: (1) if all fatty acids exist as FAMEs in the medium, extract FAMEs directly for GC/MS analysis; (2) if Tween-80 is a component in the medium, remove Tween-80 from the medium extract before the derivatization; (3) if the medium contains PF-68, precipitate PF-68 from the derivatized sample before FAME extraction; (4) if the medium is not in the categories mentioned above, simply follow medium lipid extraction and derivatization by using a large volume of medium sample, smaller quantities of high quality derivatizing reagents and extraction solvents.

For the serum-containing media (usually containing 5–10% serum), lipid composition is more complex, and the total lipid concentration in the media is in the range of tens to hundreds of mg/L level [14]. Since the interference from derivatizing reagent, solvent and Tween-80 would likely be not significant in the quantitation of lipids at concentration levels higher than tens mg/L, the sample preparation can be carried out according to procedures described in most of the published methods [8,15] and using appropriate solvent to ensure complete extraction of complex lipids from serum.

The lipids added into insect cell culture medium usually also include α-tocopherol acetate and cholesterol [3]. The α-tocopherol acetate can be quantitated in the same process if the sample is directly extracted for GC/MS analysis. However, if the medium extract is derivatized by BF_3–methanol, α-tocopherol acetate can be hydrolyzed to α-tocopherol, whose quantitation is not feasible under these experimental conditions.

4. Conclusion

The goal of the present study was to examine the interference of chemical reagents and medium components in the micro-quantitation of lipids in serum-free cell culture media, and to develop a method for the micro-quantitation of these lipids. This experiment uncovered that trace amounts of fatty acids in derivatizing agents and extraction solvents were sources of fatty acids in the micro-lipid quantitation procedure. The fatty acids derived from Tween-80 and the interference of PF-68 in the FAME extraction can be minimized by their precipitation. The results also demonstrate that micro-quantitation of FAMEs and free fatty acids in serum-free cell culture media is feasible through a careful preparation of samples, method validation and quality control.

Acknowledgements

Authors thank Chantale Beaulieu for providing expert assistance in GC/MS analyses and Marc Aucoin for critical editorial comments.

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