The BUME method: a novel automated chloroform-free 96-well total lipid extraction method for blood plasma

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Abstract Lipid extraction from biological samples is a critical and often tedious preanalytical step in lipid research. Primarily on the basis of automation criteria, we have developed the BUME method, a novel chloroform-free total lipid extraction method for blood plasma compatible with standard 96-well robots. In only 60 min, 96 samples can be automatically extracted with lipid profiles of commonly analyzed lipid classes almost identically and with absolute recoveries similar or better than what is obtained using the chloroform-based reference method. Lipid recoveries were linear from 10–100 µl plasma for all investigated lipids using the developed extraction protocol. The BUME protocol includes an initial one-phase extraction of plasma into 300 µl butanol:methanol (BUME) mixture (3:1) followed by two-phase extraction into 300 µl heptane:ethyl acetate (3:1) using 300 µl 1% acetic acid as buffer. The lipids investigated included the most abundant plasma lipid classes (e.g., cholesterol ester, free cholesterol, triacylglycerol, phosphatidylcholine, and sphingomyelin) as well as less abundant but biologically important lipid classes, including ceramide, diacylglycerol, and lyso-phospholipids. This novel method has been successfully implemented in our laboratory and is now used daily. We conclude that the fully automated, high-throughput BUME method can replace chloroform-based methods, saving both human and environmental resources.

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Lipids belong to a class of molecules with an immense structural diversity and function (1). In addition to their important role in conserving membrane integrity, they participate in many intracellular processes, such as signal transduction, apoptosis, and membrane trafficking. As a consequence, disturbances in lipid metabolism have been implicated in several diverse diseases, such as type 2 diabetes, Alzheimer disease, (2) and cancer (3). Lipid analysis has been an important area of research for several decades, and due to technological advances, the field has experienced a renaissance in the last decade. In a modern laboratory, a comprehensive lipid characterization can be performed that generates quantitative data of several hundreds of molecular lipids from several different lipid classes. This kind of analysis, often called lipidomics, is based on HPLC and mass spectrometry (MS) instrumentation. The analysis is performed unattended in the 96-well format and is fully automated.

An important component for successful analysis is the quality of the lipid extract. It is important that the lipid extract that is injected on the HPLC or infused into the mass spectrometer is pure; therefore, it is important that interfering substances and particles are removed. Inability in removing these substances might result in a high chemical background, which will have an effect on both the sensitivity and selectivity of the analysis. In contrast to fully automated and high-throughput analysis, lipid extraction is still often performed manually, involving exhaustive and time-consuming pipetting steps and hazardous solvents such as chloroform. Thus, a fully automated, chloroform-free method that can be used with standard 96-well robots would significantly improve sample throughput, as well as reduce the negative impact on health and environment. The aim of this study was to develop that method.

Abbreviations: BUME, butanol and methanol; CE, cholesteryl ester; CER, ceramide; DAG, diacylglycerol; DIPE, diisopropyl ether; FC, free cholesterol; GluCER, glucosylceramide; LacCER, lactosylceramide; LPA, lyso-PA; LPC, lysophosphatidylcholine; MTBE, methyl-tert-butyl ether; NL, neutral loss; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PIS, precursor ion scanning; PS, phosphatidylserine; SM, sphingomyelin; TAG, triacylglycerol.

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Two of the most commonly used methods for extracting and purifying lipids are the Folch (4) and Bligh and Dyer (3) procedures. Even though both of these methods are highly efficient for extracting lipids within a wide range of hydrophobicity, they are associated with important disadvantages. Both of these methods are based on chloroform, which in a mixture with methanol, is the primary solvent for solubilization. After separation of the lipids from nonlipids by creation of a two-phase system by adding a buffer, the lipids end up in the chloroform phase at the bottom of the tube. This means that for recovery of the lipid fraction, the upper water phase and the interphase, which often contains protein debris, have to be penetrated. This step can easily lead to contamination of the extract and produce clogging of the electrospray interface and HPLC system. Alternative methods free of chloroform, such as the methods published by Dole et al. (6) and Cham et al. (7), are also available. Despite generating an upper organic phase containing the lipids, these methods either are incompatible with the extraction of lipids with a wide range of hydrophobicity or result in lower recoveries. Recently, a method based on methyl-tert-butyl ether (MTBE) was published by Matyash et al. (8). Even though this method was shown to have a lipid-containing upper phase and to be well-suited for total lipid extraction, the method has a high solvent-to-sample ratio, making automation in the 96-well format challenging.

In this article, we describe a fully automated lipid extraction protocol, in which we are able to extract lipids from 96 plasma or serum samples in only 60 min. The method, which is free of halogenated solvents, is based on a mixture of butanol and methanol (BUME) for the initial extraction step. Furthermore, the method has a low solvent-to-sample volume ratio, and because it is based on organic solvents with low density, the lipids will be enriched in the upper phase, facilitating their recovery and limiting the risk for contamination prior to MS analysis. We also present an extension of the method for automated subtraction of the total lipid extract into a polar fraction and a neutral fraction.

METHODS

Materials

Butanol, heptane, ethyl acetate, methanol, ethanol, tetrahydrofuran, chloroform, dichloromethane, acetone, MTBE, and diisopropyl ether (DIME) were all of HPLC grade and attained from Rathburn Chemicals Ltd. (Walkburn, UK). Isopropanol was from Acros Organics (Pittsburgh, PA), and acetic acid was from Merck (Darmstadt, Germany). Nonradiolabeled lipid standards were from Avanti Polar Lipids (Alabaster, AL). Radiolabeled 14C glucose, 14C lysophosphatidylcholine (LPC, 1-palmitin) and 14C cholesterol ester (CE, oleate) were from Amersham Bio-Science (Little Chalfont, Bucks, UK). Radiolabeled 14C triacylglycerol (TAG, tripalmitin) and 14C diacylglycerol (DAG, dioleate) were from American Radiolabeled Chemicals Inc. (St Louis, MO), and the 14C free cholesterol (FC) was from Perkin-Elmer Life Science (Boston, MA). Ninety-six-well alumina racks for individual 1.2 ml glass vials were designed in-house, in line with the standard 96-format footprint, and they were manufactured by Mymeko Maskinteknik (Angered, Sweden). Glass vials (1.2 ml, 40 × 8 mm; product 27775/2) were from Hecht-Assistant (Sondheim, Germany). Standard serum Seronorm Lipid (freez-dried bovine serum) was from SERO AS (Billingstad, Norway), and Intralipid 20% was from Fresenius Kabi AB (Uppsala, Sweden).

Preparation of samples for method development

Three types of samples were made by dissolving freeze-dried serum in 3 ml water containing 0, 10, or 50% Intralipid (0, 2, 10% added TAG). These samples are called normal, medium, and high fat, respectively. During the solvent-screening phase of the method development, human plasma samples attained from the local biobank were pooled and aliquoted.

Development of an automated extraction protocol

In our effort to develop a rapid, robust, and efficient automated extraction procedure, a set of requirements were initially postulated (Table 1). Solvents for the initial one-phase extraction step (solvent 1), which is important for the simultaneous and rapid dissolution of lipoproteins and solubilization of lipids, were selected among low-toxic, nonchlorinated, semipolar solvents (butanol, ethyl acetate, and MTBE) and nonpolar solvents (heptane and DIME). The requirement was that the solvent, in combination with water-miscible alcohols (methanol, ethanol, or isopropanol) formed a one-phase mixture with water or plasma at a solvent:sample volume ratio less than 4:1. After mixing 300 µl of solvent 1 with 75 µl plasma, the formed mixture was inspected and evaluated against the automation criteria in Table 1. The solvents evaluated for the secondary two-phase extraction (solvent 2), for separation and purification of the lipid extract, were pure heptane and MTBE or ethyl acetate alone or in combination with heptane. Here the requirements were that addition of 300 µl solvent 2 and 300 µl buffer (1% acetic acid as model buffer) to the previously formed one-phase system would result in a spontaneous formation of a two-phase system after pipette mixing. Visual observations according to the criteria in Table 1, in combination with recoveries of a radiolabeled lipid (14C-LPC) and retention of nonlipids (14C-glucose) in the aqueous phase, guided the final selection of solvent mixtures for development of an optimized method. This method formed the basis for the BUME method.

After the selection of suitable extraction solvents using 1% acetic acid as model buffer, a chemometric approach was used for optimization of the detailed composition of the solvent-buffer system. The variables that were optimized were the amount of acetic acid (0.1–1.0%) or sodium chloride (0–3%) in the water phase and the amount of methanol in solvent 1 (butanol:methanol)

| Method Requirements |
|---------------------|
| 1. > 75 µl plasma in 96-well format |
| 2. Initial one-phase extraction with "solvent 1" giving a fine suspension |
| 3. Solvent:sample volume ratio of < 4:1 in the initial one-phase mixture |
| 4. Secondary two-phase extraction with "solvent 2" giving an upper organic phase |
| 5. Organic phase 40–60% of total volume |
| 6. Total volume of sample, solvents, and buffer within 1000 µl |
| 7. Spontaneous phase separation—no centrifugation |
| 8. Fully automated steps for aspirating, mixing, and dispensing |
| 9. Possible to perform three repeated automated extraction steps 10. < 90 min extraction time per 96-well plate |

*Enables efficient and reproducible pipette and vortex mixing.*
and ethyl acetate in solvent 2 (heptane:ethyl acetate). The evaluation was performed using Modde software version 7.0 (Umetrics, Umeå, Sweden).

After finalizing the protocol in terms of solvent selection and ratios, the method was set up on a Velocity 11 Bravo pipetting robot (Agilent Technologies, Santa Clara, CA). Robot parameters, such as speed and position of the different aspiration and dispensing steps, were optimized to ensure high recoveries and a robust protocol capable of extracting sample volumes ranging from at least 10–75 µl without modifying the robot protocol parameters. The performance of the optimized automated BUME method was evaluated against a manually performed Folch method in terms of lipid class recoveries, reproducibility, composition of molecular species in several lipid classes, and the capacity to extract samples rich in fat. The automated BUME method was further tested for sample volume range and linearity. Finally, benefits on health and environment were identified for the BUME method versus the Folch method.

The reference method: a Folch-based extraction protocol
Lipids were extracted using a modified Folch procedure (4) commonly used in our laboratories. Briefly, 500 µl methanol was added to 60 µl Seronorm Lipid in a 10 ml glass tube. After 10 min of vortex mixing, 1,000 µl chloroform was added. After another 10 min of vortex mixing, 300 µl of 20 mM acetic acid was added, and the two-phase system was again vortex mixed for 10 min. After 5 min of centrifugation at 1,000 g, the lower organic phase was transferred to a new tube, and the water phase was washed with 500 µl chloroform. Finally, after 10 min of vortex mixing and 5 min of centrifugation to induce phase separation, the organic phases were pooled, evaporated, and reconstituted in chloroform:methanol (2:1). The lipid extracts were stored at −20°C until further analysis.

Quantification of endogenous lipids
Lipid extracts of Seronorm Lipid spiked with 0, 10, and 50% Intralipid were evaporated to dryness under a stream of nitrogen at 30°C, reconstituted in heptane:isopropanol (9:1), and injected into a normal-phase HPLC system. CE, TAG, and FC were separated and quantified using ELS detection, according to previous work (9). The HPLC system was used in combination with a fraction collector for purification of DAG and ceramide (CER) for further analysis.

Quantification of DAG, CER, phosphatidylcholine (PC), sphingomyelin (SM), and LPC was performed using MS. For PC, SM, and LPC, total lipid extracts were reconstituted in chloroform:methanol (1:2) with 5 mM ammonium acetate with internal standards containing C17:0 fatty acids. The reconstituted extracts were then infused directly into a QTRAP5500 instrument (AB Sciex, Concord, Canada) equipped with a robotic nanoflow ion source, TriVersa NanoMate (Advion Biosciences, Ithaca, NY). The characteristic phosphocholine fragment m/z 184.1 was selected for precursor ion scanning (PIS) of PC, SM, and LPC in positive ion mode according to previous work (10, 11).

After purification using the normal-phase HPLC system, the DAGs were also reconstituted in chloroform:methanol (1:2) with 5 mM ammonium acetate containing 1,2-DAG 17:0/17:0 as internal standard. This solution was analyzed using the TriVersa NanoMate/QTRAP5500 setup, and the DAGs were monitored in positive ion mode by neutral loss (NL) of the most common fatty acids (12).

Although quantified as a lipid class using the HPLC system, profiles of the molecular CE were obtained from the total lipid extract using the TriVersa NanoMate/QTRAP5500 setup. Here the characteristic dehydrocholesterol fragment m/z 369.3 was selected for PIS of CEs in positive ion mode (13).

Raw data generated from the TriVersa NanoMate/QTRAP5500 setup was processed using the LipidView software (AB Sciex, Concord, Canada).

The CERS obtained from the HPLC purification were analyzed using reversed-phase HPLC coupled to a triple quadrupole Quattro Premiere mass spectrometer (Waters, Milford, MA) as previously described (14).

Estimation of absolute lipid recoveries
An estimation of the absolute recoveries of neutral lipids was made by spiking 60 µl of Seronorm Lipid with radiolabeled CE, TAG, FC, and DAG (Table 2). After BUME extraction, the radioactivity was measured in the organic phase. As a reference (100% recovery), 60 µl of Seronorm Lipid was extracted, and then same amount of radioactivity was spiked into the lipid extract to obtain identical matrix effects. The radioactivity was measured using a Wallac 1409 scintillation counter (Wallac, Turku, Finland).

For estimation of absolute recoveries of phospholipids and sphingolipids, a set of molecular lipids from 12 different lipid classes were spiked into 60 µl of Seronorm Lipid and extracted using both the Folch and BUME procedures (Table 3). Before analysis, another set of molecular lipids was added, which was used for quantification. As a reference (100% recovery), 60 µl of Seronorm Lipid was extracted and then spiked with both sets of standards. Analysis of the lipid extracts was made using direct infusion on the TriVersa Nanomate/QTRAP 5500 setup described above. Detection of the spiked lipids was made using NL and PIS experiments. See Table 3 for further information.

Statistical evaluation
Comparisons between groups were performed using Student t-test. P < 0.05 was considered significant.

RESULTS
Optimized solvents and buffers for automated total lipid extraction
Several solvents were tested to investigate whether they fulfilled the automation requirements postulated in Table 1. Initial visual observations of the tested solvent mixtures indicated very good properties for butanol:methanol (BUME) mixtures for the initial one-phase extraction. When a mixture of butanol:methanol (5:1 to 1:1) was added to frozen plasma in the 96-well glass vials, a one-phase system with a fine suspension formed that was easy to mix without clogging the pipette tips. BUME mixtures as solvent 1 also worked very well in combination with solvent 2 (pure heptane and MTBE, or ethyl acetate alone or in combination with heptane) and the buffer and fulfilled all requirements postulated in Table 1 for automation.

| Lipid                        | Recovery (%) |
|------------------------------|--------------|
| 14C cholesteryl ester (18:1) | 95 ± 7.0     |
| 14C cholesterol             | 102 ± 2.8    |
| 14C triacylglycerol (16:0/16:0/16:0) | 93 ± 3.6  |
| 14C diacylglycerol (18:1/18:1) | 94 ± 3.2    |

Values are mean ± SD.
From the initial observations, it became clear that the capacity of solvent 1 to dissolve water is important to fulfill the postulated criteria of having a solvent:sample ratio <4 in the initial one-phase system. We therefore investigated the ability of methanol-containing mixtures of butanol, MTBE, or chloroform to dissolve water while maintaining a one-phase system. The data (supplementary Fig. I) showed that the proportion of methanol required increased with decreasing polarity of the solvent (butanol < MTBE < chloroform). Less than 15% methanol was required for 4 vol of butanol:methanol to mix with water, but almost 50% was required for MTBE:methanol.

Addition of solvent 2 and buffer to plasma mixed with BUME mixtures generally resulted in a rapid and spontaneous phase separation after mixing. When heptane was used alone or in combination with ethyl acetate or MTBE, phase separation occurred within 5 min with a distinct interface. The use of these solvents also resulted in a lipid extract volume that facilitated efficient transfer to new vials and repeated extractions. Measurements of volumes of the aqueous phase and organic phase indicated that buffer and methanol were combined in the aqueous phase and that butanol and solvent 2 were combined in the organic phase, regardless of the composition of solvent 2 as long as heptane was present.

To further optimize the proportion of methanol in butanol (the BUME mixture), the proportion of ethyl acetate in heptane, and the amount of sodium chloride or acetic acid in the buffer, a chemometric approach was used. This evaluation showed that for a BUME mixture, a ratio 1:3 of methanol in butanol gave the highest recoveries of LPC. As illustrated in supplementary Fig. II, using 25% methanol in butanol resulted in a 90% recovery for LPC, compared with a modest 55% recovery when 50% methanol was used in the BUME mixture.

The solvent optimization experiments also showed that the composition of solvent 2 was less critical. However, the recoveries of LPC were increased from less than 85% with pure heptane to almost 90% with 25% ethyl acetate (supplementary Fig. III). Increasing the ethyl acetate ratio above 25% did not increase the lipid recoveries, but the coextraction of glucose rapidly increased from about 1% glucose with 25% ethyl acetate to 10% glucose with 100% ethyl acetate. Heptane:ethyl acetate (3:1) was selected for the final BUME protocol.

The results from the chemometric optimization also showed that buffer composition was not critical, as there were only small differences with respect to lipid recoveries between the various buffers tested (data not shown). One percent (1%) acetic acid in water was selected because a high ionic strength in general as well as a low pH should be favorable for the extraction of polar acidic lipids that may be difficult to recover. However, as the relatively high amount of acetic acid might have consequences on lipid stability, especially for the acid-labile plasmalogens, we tested this by spiking the plasmalogen PC-O 18:0/20:4 to a Seronorm Lipid sample and extracting it with 0%, 0.1%, or 1% acetic acid. The result did not show any significant difference in the extraction yield between the different conditions and we therefore draw the conclusion that there is no major lipolysis caused by the acidic acid (supplementary Fig. IV). These data are further supported by the high absolute extraction yields for the plasmalogens using the BUME procedure (Table 3).

Similar to the BUME mixtures, MTBE:methanol (1:1) as solvent 1 formed a mixture with plasma that was easily mixed without clogging the tips. However, in contrast to the good results for BUME, a mixture of MTBE:methanol (1:1) as solvent 1 did not result in spontaneous phase separation, even when heptane was included in solvent 2, and therefore, it required centrifugation. Furthermore, MTBE evaporated rapidly from trays and vials and made automation more difficult and less reproducible. The results also showed that the recovery of LPC was only 50% with MTBE:methanol (1:1) as solvent 1 and MTBE as solvent 2, similar to the observation made for BUME mixtures with 50% methanol in butanol (supplementary Fig. II).

**TABLE 3. Absolute recoveries of phospholipids and sphingolipids**

| Standard 1* | Standard 2* | MS Method  | BUME | Folch |
|------------|------------|-----------|------|-------|
| PC 17:0/17:0 | PC 17:0/20:4 | +PIS 184.1 | 97 ± 3.3 | 98 ± 3.2 |
| PE 17:0/17:0 | PE 17:0/20:4 | +NL 141.0 | 97 ± 2.9 | 102 ± 4.6 |
| PS 17:0/17:0 | PS 17:0/20:4 | +NL 185.0 | 99 ± 8.0* | 82 ± 5.1 |
| PG 17:0/17:0 | PG 17:0/20:4 | -PIS 269.2 | 94 ± 6.1* | 84 ± 1.5 |
| PA 17:0/17:0 | PA 17:0/20:4 | +NL 115.0 | 78 ± 3.5 | 71 ± 6.7 |
| P CO 18:0/20:4 | P CO 18:0/18:1 | -PIS 281.2 | 91 ± 4.1 | 92 ± 3.2 |
| SM 17:0 | SM 10:0 | +PIS 184.1 | 97 ± 3.7 | 101 ± 5.8 |
| CER 18:0 | CER 17:0 | +PIS 264.2 | 92 ± 4.2 | 91 ± 3.4 |
| GluCER 16:0 | GluCER 12:0 | +PIS 264.2 | 91 ± 5.7 | 94 ± 3.8 |
| LacCER 16:0 | LacCER 12:0 | +PIS 264.2 | 91 ± 3.1 | 93 ± 3.2 |
| LPA 17:0 | PA 17:0/20:4 | +NL 115.0 | 69 ± 7.7* | 23 ± 4.0 |
| LPC 17:0 | PC 17:0/20:4 | +PIS 184.1 | 88 ± 3.2 | 92 ± 3.9 |

*Sixty microliters of Seronorm Lipid was used as sample matrix. Values are mean ± SD (n = 6). *P < 0.05 versus Folch.

*Phospholipids and sphingolipids were measured using precursor ion scanning (PIS) and neutral loss (NL) experiment during a constant nano-flow infusion. See Methods for further information.
The use of more lipophilic alcohols (ethanol and isopropanol) instead of methanol in the initial one-phase extraction generally resulted in lower recoveries of LPC or led to the incomplete fulfillment of the requirements postulated in Table 1. DIPE as an alternative to heptane in solvent 2 did not deliver results different from or better than heptane (data not shown).

Protocol for the rapid automated total lipid BUME extraction method using a 96-well robot

A robot protocol was defined based on the automation criteria and optimized solvents and buffers. This protocol, which is outlined in Fig. 1, was developed on a Velocity 11 Bravo robot. However, the protocol can be adapted to any standard 96-well robot that can work with 200 µl tips with at least seven pads for 96-well plates (sample, lipid extract, solvent 1, solvent 2, buffer, sample tips, and solvent tips). A picture showing the robot setup is given in supplementary Fig. V. The total run time is 60 min per plate. However, because the method is fully automated, only 15 min of manual work is required for each 96-well plate. This means that during a normal work day, more than 600 samples can be extracted with only 90 min of manual work.

START

10-100 µl frozen plasma
+ 300 µl butanol/methanol
(3:1)

Integrated vortex mixing 10 min

Add 150 µl
heptane/EtAc (3:1)

Mix 5 min with robot

Add 300 µl
1% acetic acid (aq)

Mix 5 min with robot *
Gravitational separation 5 min
Transfer 360 µl upper organic phase

Add 320/250* µl heptane/EtAc
(3:1) to water phase

Mix 5 min with robot *
Gravitational separation 5 min
Transfer 320/200* µl upper organic phase

* Mixing occurs at dual heights
* First/second cycle

Fig. 1. Extraction protocol for the developed BUME method. The extraction, which takes 60 min, is fully automated and performed in 1.2 ml glass vials in a 96-well format. EtAc, ethyl acetate.

For comparison, only 60 samples per day can be extracted in our lab using the manual Folch procedure.

Comparison of lipid recoveries between the BUME and Folch procedures

Endogenous lipid class profiles. To compare the BUME and Folch procedures, endogenous lipids were quantified in samples with varying levels of triacylglycerol content. For this purpose, 60 µl of Seronorm Lipid containing 0, 10, or 50% Intralipid was extracted (normal, medium, and high fat, respectively), and endogenous lipids were quantified. As can be seen in Fig. 2, the two extraction methods resulted in identical recoveries for all high abundance plasma lipids (CE, TAG, FC, PC, and SM) as well as for less abundant, biologically important lipids (CER and DAG). The only significant difference between the two methods was an approximate 5–10% reduction in the measured extraction yield for endogenous LPC in the normal and high-fat samples when using the BUME procedure.

Endogenous molecular lipid profiles. By using direct infusion of the total lipid extract on the TriVersa NanoMate/QTRAP5500 setup, we also generated profiles of molecular lipids for several lipid classes. Fig. 3 presents profiles of molecular lipids from four lipid classes from the medium fat samples. The identical profiles observed for the automated BUME and manual Folch procedures indicate that the automated BUME method gives accurate profiles of molecular lipids in both the hydrophobic and hydrophilic ends of the polarity spectrum.

Absolute extraction yields. An estimation of absolute extraction yield was made by using a combination of radiolabeled and nonendogenous lipid standards. For glucosylceramides (GluCER) and lactosylceramides (LacCER), endogenous lipids were used. However, as the added amount was much higher than the physiological amount, more than 99% of the signal was derived from the added lipid (data not shown).

The results showed high extraction efficiencies (>90%) of the neutral lipids CE, TAG, FC, and DAG for the BUME method (Table 2). For these lipids, we did not test the absolute recovery using the Folch procedure. However, from the experiments quantifying endogenous lipids in Seronorm Lipid, the BUME and Folch procedures gave comparable extraction yields (Fig. 2).

The results also showed high and comparable extraction yields for the sphingolipids and nonacidic phospholipids PC and phosphatidylethanolamine (PE) with absolute recoveries greater than 90% for both the BUME and Folch procedures (Table 3). In contrast, for the acidic phospholipids phosphatidylycerine (PS) and phosphatidylglycerol (PG), the BUME method was superior. For phosphatidic acid (PA), which is the most acidic phospholipid, both the BUME and Folch procedures showed relatively low extraction efficiencies (70–80%). The most striking difference between the two methods was observed for the lyso-PA (LPA) lipid class in which Folch showed a 23% recovery compared with 69% for the BUME procedure.
In addition to the lipids, we used radiolabeled glucose to estimate the ability of the methods to retain polar substances in the aqueous phase. Both the Folch and BUME procedures resulted in an approximately 99% retention of the labeled glucose in the aqueous phase. There were no differences observed between the two methods in terms of ion suppression or background signals using MS (data not shown), and extracts from both methods worked well with the Nanomate interface. In conclusion, these findings indicate very similar extraction properties for BUME and Folch procedures, with significantly higher recoveries for the most acidic polar lipids with the BUME procedure.
on shoulders and neck as well as the potential exposure to chloroform following manual Folch extraction is reduced to zero. From an environmental aspect, low-toxic solvents replace chloroform, and smaller amounts of solvents are used overall. Taken together, the novel BUME method offers a significant step forward in a modern lipid laboratory in terms of economy, health, and environment.

Further extraction steps separate neutral and polar lipid classes

In addition to the described BUME method, we developed an automated liquid-liquid extraction method for the separation of neutral and polar lipid classes. This extraction starts with a dry total lipid extract to which 150 µl of heptane:methanol (98:2) is added. The solution is mixed using the integrated orbital mixer for 10 min, and then 300 µl methanol:buffer (95:5) and another 300 µl heptane:methanol (98:2) is added. The buffer contains 1% of concentrated (23%) ammonia. After pipette mixing and spontaneous phase separation, the heptane phase containing the neutral lipids is removed, and the aqueous methanol phase is washed twice with 300 µl of heptane:methanol (98:2). Evaluation of this extraction method

The BUME method is compatible with a high sample-to-solvent ratio

An important step in the lipid extraction procedure is the initial one-phase extraction in which, in this case, a butanol:methanol (3:1) mixture was added to the sample matrix. A low solvent-to-sample ratio might lead to the formation of a two-phase system, which will result in reduced extraction yields. To investigate the possible limits of sample volume for accurate extraction, we extracted different volumes of Seronorm Lipid containing 10% of Intralipid (medium fat). The results showed that the automated BUME method is linear when extracting 10–100 µl (30:1 to 3:1 solvent-to-sample volume ratio) (Fig. 4). The DAGs and CERs were not investigated in this respect. However, based on their intermediate polarity and high recoveries (>90%), similar results can be expected for these lipid classes.

The BUME method is superior to Folch in terms of costs, health, and environment

The BUME method requires about 50 times less labor cost in terms of extraction time and the consequent release of time for lipid research. Human costs related to the load of shoulders and neck as well as the potential exposure to chloroform following manual Folch extraction is reduced to zero. From an environmental aspect, low-toxic solvents replace chloroform, and smaller amounts of solvents are used overall. Taken together, the novel BUME method offers a significant step forward in a modern lipid laboratory in terms of economy, health, and environment.
have been developed in our lab (15, 16). However, factors such as a high solvent-to-sample ratio, a lower lipid-containing organic phase, and the need for centrifugation resulted in a nonrobust, time-consuming, semiautomated process. This concern, together with environmental awareness, contributed to the decision to develop a method based on nonhalogenated, low-toxic solvents, which resulted in the BUME method.

In both the Folch and the Bligh and Dyer procedures, chloroform is used with methanol for the initial one-phase dissolution and simultaneous solubilization of the sample matrix lipids. This step is important for biofluids and critical for tissue samples. To avoid halogenated solvents, we identified and optimized a combination of butanol and methanol for this purpose. Similar to chloroform, butanol dissolves even very lipophilic lipids and is poorly soluble in aqueous samples. However, when mixed with methanol, its water solubility increases, and the ability to form polar as well as nonpolar interactions makes the combination of butanol and methanol suitable for one-phase lipid extraction from aqueous samples. We also noted that the denaturation of proteins as a result of the addition of the BUME mixture resulted in a fine suspension that was easy to mix using the automated setup. As this was especially true when the BUME mixture was added to frozen plasma, thawing the plasma samples prior to extraction can be avoided. This is beneficial as it might reduce the ex-vivo modification of metabolically fragile lipids.

DISCUSSION

In this study, we developed a novel and fully automated method for performing total lipid extraction from plasma or serum using a basic 96-tip robot. The method, which is based on nonhalogenated solvents, is performed in 60 min. It shows almost identical extraction yields compared with the previous gold standard methods, with major advantages with respect to sample throughput and volume range, as well as economic, health, and environmental aspects.

Within the field of lipid analysis, extraction is an often overlooked but highly important procedure. The development of automated lipid extraction protocols can save time and resources to allow more focus on data processing and analysis. Moreover, as manual extraction is often associated with nonergonomic pipetting and extensive handling of organic solvent, automation also has health benefits. Semiautomated extraction protocols based on the Folch procedure have been described by others and have been developed in our lab (15, 16). However, factors such as a high solvent-to-sample ratio, a lower lipid-containing organic phase, and the need for centrifugation resulted in a nonrobust, time-consuming, semiautomated process. This concern, together with environmental awareness, contributed to the decision to develop a method based on nonhalogenated, low-toxic solvents, which resulted in the BUME method.

In both the Folch and the Bligh and Dyer procedures, chloroform is used with methanol for the initial one-phase dissolution and simultaneous solubilization of the sample matrix lipids. This step is important for biofluids and critical for tissue samples. To avoid halogenated solvents, we identified and optimized a combination of butanol and methanol for this purpose. Similar to chloroform, butanol dissolves even very lipophilic lipids and is poorly soluble in aqueous samples. However, when mixed with methanol, its water solubility increases, and the ability to form polar as well as nonpolar interactions makes the combination of butanol and methanol suitable for one-phase lipid extraction from aqueous samples. We also noted that the denaturation of proteins as a result of the addition of the BUME mixture resulted in a fine suspension that was easy to mix using the automated setup. As this was especially true when the BUME mixture was added to frozen plasma, thawing the plasma samples prior to extraction can be avoided. This is beneficial as it might reduce the ex-vivo modification of metabolically fragile lipids.

Isopropanol is an alcohol frequently used, often in mixtures with alkanes, for the initial one-phase extraction (6, 17). However, as we noted during method development, the distribution of this alcohol between the lipid
phase and the aqueous phase was very sensitive to the volumes and compositions of the solvents and buffers used. In contrast, we observed from the robust and reproducible volumes of the lipid and aqueous phases that the BUME mixture, regardless of the composition of the second solvent, always separated into its constituents, resulting in a methanol-enriched aqueous phase and a butanol-enriched lipid phase. The ability of the BUME mixture to separate in this manner during the two-phase extraction step is an important feature for high lipid recovery and low lipophilicity of the formed aqueous phase. For butanol, in contrast to MTBE or chloroform and more lipophilic solvents, only a small volume of methanol is required to make butanol water-miscible in the desired solvents:sample volume ratios of less than 4:1. The aqueous phase remains highly polar after phase separation, containing only a small amount of methanol and very little butanol. This probably explains the good recoveries of polar lipids even when using small volumes.

The purpose of the addition of the second solvent mixture is to separate lipids, extracted from the sample matrix by the BUME mixture, from nonlipids in the aqueous phase by formation of a distinct two-phase system. The distribution of lipids between the lipid phase and the aqueous phase is determined by the ability of the lipid phase to dissolve both polar and nonpolar lipids efficiently. This requires a rather polar lipid phase with a high content of butanol, whereas the aqueous phase should be as polar as possible with a high content of water to retain only nonlipids, such as glucose and amino acids. We observed that heptane alone (forming a mixture with butanol) resulted in high recoveries of all investigated lipids but that addition of ethyl acetate to heptane was even better, in particular for phospholipids.

In the method described, we used a robot equipped with a standard 200 µl tip head and a standard orbital shaking system, fully integrated in the hardware and software. Because the robot cannot mix samples and solvents as vigorously as a bench-top vortex mixer and cannot separate the often-formed emulsion following such a procedure by centrifugation, robot mixing techniques and mixing times become important. The mixing technologies utilized were orbital shaking (mild vortex mixing) and repeated aspiration/dispensing steps (pipette mixing). Vortex mixing was used only initially after adding 300 µl BUME to the frozen plasma sample, whereas pipette mixing is the method of choice when larger volumes are present in the tubes. The pipette mixing is done repeatedly at dual heights; aspirating 100 µl of the upper part of the lipid extract at the higher level and dispensing it just above the surface of the lower phase at a lower level. Mixing takes place both in the pipette tip and in the tube, where the aspirated solvent is dispersed like a jet beam into the aqueous layer, forming small droplets and thereby a large surface for efficient two-phase lipid extraction. Depending on the actual robot used and tip design, the number of pipette mixing cycles and the dispensing speed must be optimized when setting up the BUME protocol. During a manual procedure, reextraction of the water phase is labor intensive and, therefore, normally performed only once. During automated extraction, however, several wash steps can be included. In the BUME method, we washed the water phase two times, and the three organic aliquots are pooled and evaporated. This enabled us to reach high extraction yields, despite performing the extraction in a small 1.2 ml glass vial.

To test the developed method, we made comparisons to the Folch method, which is one of the gold-standard methods in the field. Initially we quantified endogenous lipids in serum samples spiked with increasing amount of triglycerides ( Intralipid). The addition of extra triacylglycerols was made because a previous comparison between the Folch and Bligh and Dyer procedures indicated that a high amount of triglycerides can result in loss of robustness and reduced extraction yields (18). Our results showed almost identical extraction recoveries between the BUME and Folch procedures independent of the triglyceride content for all tested lipid classes, with the exception of LPC. This lipid was less efficiently extracted using the BUME method. However, the difference in extraction yield between the Folch and BUME procedures was smaller using radiolabeled LPC (using liquid scintillation counting) than it was using endogenous LPC (using MS), which may be explained partly by the fact that endogenous LPC may result from lipolysis of the more abundant PC during sample preparation, which is not the case for 14C-LPC. The manual Folch protocol is potentially more prone to lipolysis as it takes more time and is performed at a higher temperature than the BUME procedure. In the BUME procedure, solvents are taken directly from the freezers and are used immediately with frozen samples.

We continued our comparison to Folch by testing the extraction efficiency for the developed BUME method for a number of different lipid classes. Both the Folch and BUME methods showed high and similar recoveries for the neutral lipids, sphingolipids, and nonacidic phospholipids. However, the BUME procedure showed better recoveries for the acidic lipids PG and PS. It has previously been described that the original Folch procedure is not optimal for extracting acidic phospholipids and that the addition of acetic acid can be used to increase the recoveries of acidic lipids in chloroform/methanol-based extraction procedures (19, 20). However, despite using a modified Folch protocol containing 20 mM of acetic acid, the BUME protocol showed better recoveries of the acidic phospholipids. It might be that the additional amount of acetic acid in the BUME procedure [174 mM (1%) compared with 20 mM for the Folch procedure] led to higher recoveries. BUME also showed much higher recoveries of LPA. The poor recovery of this lipid class using the Folch procedure has previously been reported, and several publications suggested the use of butanol for extraction of this lipid class (21–23).

An important strength with the BUME method is its low solvent-to-sample volume ratio. We tested the linearity of the method and observed that efficient and robust extraction recoveries could be obtained even at 100 µl of sample (3:1 solvent-to-sample volume ratio in the initial one-phase

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extraction). This is compared with the Folch method that uses a solvent-to-sample volume ratio of 20:1, which substantially limits the sample volumes compatible with the automated setup.

Recently a new manual, chloroform-free method using a combination of MTBE and methanol was published (8). The authors showed that by using this solvent combination for the initial one-phase extraction, high recoveries of a wide range of lipid classes could be attained. This method appears to be a good alternative to the Folch method for manual extractions. A limitation of this method, however, was the use of a very high solvent-to-sample volume ratio (32.5:1) during the initial one-phase extraction, which makes automation in a 96-well format challenging. During the solvent selection phase of the development of the BUME method, we observed that MTBE:methanol for the initial one-phase extraction step required almost 50% methanol in MTBE to allow less than 4:1 solvent-to-sample ratios. We also observed that spontaneous phase separation did not occur and thus centrifugation was required when MTBE was used as the second solvent for the two-phase separation. Also, only 50% recovery of lyso-PC was found, probably due to the large amount of methanol required, with an automated MTBE:methanol method.

Lipidomics is an emerging field with the ambitious task of comprehensively characterizing a large number of lipids from a single sample. Often such a characterization covers lipids with a broad array of polarity, and it is impossible to find a single method for accurate high-yield extraction of all lipids of interest. Therefore, several extractions will have to be performed, either in series or in parallel. Recently Ejsing et al. comprehensively analyzed the yeast lipidome (24). As this characterization covered lipids with a wide range of polarity, they performed an initial chloroform-based extraction of the neutral lipids and a subsequent reextraction of the remaining phase to isolate the more hydrophilic substances. Similar approaches can be taken for extraction of the sphingolipidome as described by Shaner et al. (25). For these applications, automated processes are extremely valuable to reduce time-consuming multiple extraction procedures. For this reason, we also describe, in addition to the BUME method, an automated method for dividing the final lipid extract from the BUME procedure into nonpolar and polar subfractions. This way of cleaning up and reducing the sample matrix is highly valuable, especially when performing shotgun analysis (i.e., direct infusion) of lipid extracts. For example, if the phospholipids in adipose tissue were to be studied, it would be highly desirable to remove the majority of the TAG prior to shotgun analysis. This would reduce contamination of the ion source and reduce ion suppression, which would otherwise reduce the sensitivity of the analysis.

In this study, we have focused on relatively hydrophobic lipids. However, we believe that the BUME method might be extended to extraction of highly polar lipids polar, such as sphingosine bases and gangliosides, probably retained in the aqueous phase. Today these lipid classes are mainly purified using a one-phase extraction (25, 26). Therefore, accurate extraction of these lipid classes might be achieved by using the initial one-phase extract for further analysis without performing the two-phase extraction step or by adding extraction steps on top of the BUME method. It may well be possible to use a solvent mixture based on a high proportion of butanol for additional automated extractions of very polar lipids in the aqueous phase from the original BUME method, similar to what has previously been described (27). However, the development of a method for the extraction of very polar lipids lies outside the scope of this report and needs to be investigated further.

In this study, we have focused on the extraction of plasma lipids. However, much of the development was intended to expand the methodology into tissue extraction. This work has started already, and preliminary experiments based on the BUME protocol have generated promising data (unpublished data).

In conclusion, we present here a novel and fully automated method for total lipid extraction from blood plasma or serum. We have highlighted the benefits of using automated extraction protocols, which increase throughput as well as reduce strains on both environmental and personal health. Automation also provides a tool for performing the labor-intense, sequential extraction protocols often required for studies using a lipidomics approach. Because the method involves low-toxic solvents and has been shown to have high extraction recoveries for all the investigated lipids, we believe that this method could replace the old chloroform-based methods often used today and that the BUME method could become the method of choice for the modern lipid laboratory.

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