

# Advances in Solvent Extraction and Analysis



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## ABSTRACT

Accelerated solvent extraction is a high-temperature, high-pressure extraction technique that is widely used for various extraction protocols in food analysis. Soxhlet extraction, one of the oldest solvent extraction methodologies, is typically pursued at or near atmospheric pressure and with only slightly elevated temperatures. Extractions performed under soxhlet conditions take longer than those typically pursued with a pressurized solvent extraction technique such as accelerated solvent extraction. Solvent consumption and extraction time is significantly reduced as compared to soxhlet extraction. Extractions at higher temperatures and pressures also allow faster extraction of analytes relative to conventional liquid-liquid (L-L) based extraction techniques.

A new extraction method for acid and base hydrolyzed samples is presented here. Additionally, a new partial parallel mode of extraction is discussed. Recovery data for a variety of food samples is shown here for the above methods, along with solvent usage and extraction time.

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## INTRODUCTION

### Acid/Base Hydrolysis—Extraction of Food Samples for Lipid Analysis

Some foods contain lipids complexed with carbohydrate or proteins. These samples require pretreatment with concentrated reagents. The purpose of hydrolysis is to release bound lipids.

Acid hydrolysis with hydrochloric acid breaks down the lipid-carbohydrate bonds and hydrolyzes the proteins and starch, breaking chemical bonds and liberating the fat to facilitate extraction. Samples run by the acid hydrolysis method include cheese products and cooked foods such as cereals, bread, cookies, chips, mayonnaise, and meat.

Base hydrolysis with ammonium hydroxide weakens lipid-protein bonds, disrupts the casein micelles, and breaks up fat emulsions. Samples run by the base hydrolysis method include dairy products such as milk, half and half, heavy whipping cream, sour cream, cream cheese, and condensed milk.

The Association of Official Analytical Chemists International (AOAC) method (996.06) uses acid or base hydrolysis of food followed by the Mojonnier method for L-L extraction. The lipid analysis can be pursued by gravimetry and GC/GC-MS after converting the fat into fatty acid methyl ester (FAME) form.

### Mojonnier Method for Acid/Base-Hydrolyzed Food Samples

The Mojonnier method is an L-L extraction protocol requiring intimate mixing of the two solvent phases (aqueous and organic) to aid extraction of lipids. The acid/base fractions move into the aqueous phase while the fat moves into the organic phase during the extraction process. The Mojonnier method is typically a manual extraction process that requires constant shaking of the concentrated acid and base fractions. It could pose a safety hazard, and is labor-intensive with limited throughput. The technique relies on manual separation of the two phases by visual examination, and hence is subject to high variance.

One serious issue with the original method (AOAC 996.06) when implemented with the Mojonnier flask is that it is difficult to ensure that the entire sample remains at the bottom of the flask, particularly with viscous samples such as mayonnaise. Incomplete hydrolysis may occur if the samples are not fully immersed into the acid or base reagent. This may lead to poor recoveries of fat. In this work, a straight-walled container was used to pursue the hydrolysis, transferring the contents to the Mojonnier flask for extraction.

## ASE Extraction for Running Acid/Base-Hydrolyzed Samples

Previous generation Accelerated Solvent Extractor (ASE®) instruments used stainless steel components that are adversely effected by the acid or base reagents. The effects include blackening of the components such as cells, frits, and filters, as well as clogging of the tubing and valves. These effects resulted in significant down time of the instrument.

The above discussed issues are addressed in the current ASE instruments via:

- a) pH-hardened pathways that use acid/base resistant material in the flow pathways;
- b) ASE Prep CR resin formulation that can neutralize the acid/base reagents during in-cell extraction.

## New ASE Method for Acid/Base-Hydrolyzed Food Samples

### Protocol

- ASE Prep CR Na form resin is used to remove acid.
- ASE Prep CR H form resin is used to remove base.
- ASE Prep DE (diatomaceous earth) is used to absorb water.
- This combination can neutralize and absorb interferences from the acid/base hydrolyzed sample
  - Add acid hydrolyzed sample into the ASE Prep CR Na form resin and ASE Prep DE, load in a 100 mL zirconium cell, and begin in-cell extraction.
  - Add base hydrolyzed sample into the ASE Prep CR H form resin and ASE Prep DE, load in a 100 mL zirconium cell, and begin in-cell extraction.

### Advantages

- Supports extraction in the presence of acids/bases and achieves good recovery of lipid samples
  - Removes acid/base, thereby limiting exposure to acid/base
- Prolongs life of various components, e.g., cell, cell enclosure, tubing, frits, etc.
- Limits the technician's exposure to acid/base
  - Eliminates the need to shake the concentrated acid/base container

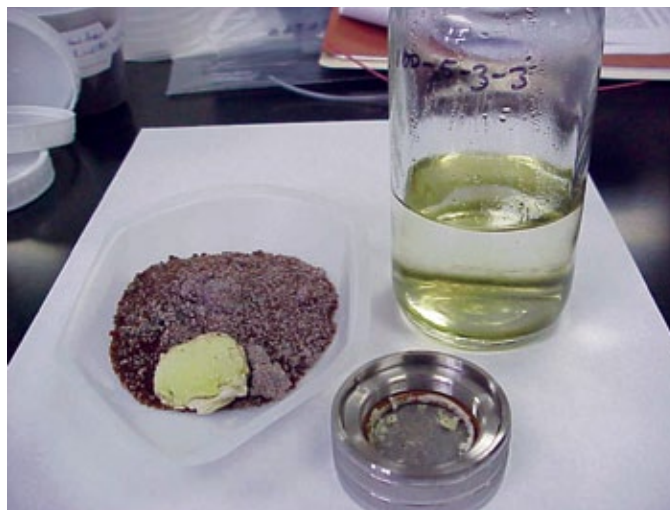


Figure 1. This figure shows damaged filter paper, acid residue at the bottom of the bottle, and a stained frit due to contact with the acid.



Figure 2. Due to the complete neutralization and absorption of the acidic sample by the resin method, no coloration is observed on the frit or on the filter.

## EXPERIMENTAL CONDITIONS

### Typical Acid/Base-Hydrolysis Conditions

#### GC/GC-MS FAME Analysis

- Food samples (variable weight, to yield 100–200 mg fat)
- Mix with 2 mL ethanol, 100 mg pyrogalllic acid, 2 mL triglyceride internal standard (5 mg/mL in  $\text{CHCl}_3$ ), and 10 mL of 8 M HCl; heat at 70–80 °C for 40 min with moderate agitation for acid hydrolysis.
- Mix with 2 mL ethanol, 100 mg pyrogalllic acid, 2 mL triglyceride internal standard (5 mg/mL in  $\text{CHCl}_3$ ), 4 mL water, and 2 mL of 58%  $\text{NH}_4\text{OH}$  (28–30%  $\text{NH}_3$  content); heat at 70–80 °C for 10–20 min with moderate agitation for base hydrolysis.

#### Gravimetric Analysis

- Food samples (variable weight, to yield 100–200 mg fat)
- Mix with 2 mL ethanol and 10 mL of 8 M HCl; heat at 70–80 °C for 40 min with moderate agitation for acid hydrolysis.
- Mix with 2 mL ethanol, 4 mL water, and 2 mL of 58%  $\text{NH}_4\text{OH}$  (28–30%  $\text{NH}_3$  content); heat at 70–80 °C for 10–20 min with moderate agitation for base hydrolysis.

### Sample Preparation Conditions

The extraction of acid- or base-hydrolyzed food samples were pursued in a 100 mL Zirconium extraction cell. The bottom cell end cap was installed and a filter was inserted. A plug of either Na or H form ASE Prep CR resin was added at the bottom of the cell. The acid-hydrolyzed sample was added into the ASE Prep CR Na form resin and ASE Prep DE in a mortar, mixed by pestle, then loaded into a 100 mL zirconium cell. Similarly, the base-hydrolyzed sample was added into the ASE Prep CR H form resin and ASE Prep DE in a mortar, mixed by pestle, then loaded into a 100 mL zirconium cell. Another plug of either Na or H form resin was added to top off the cell and the top end cap was affixed. The cell was ready for in-cell extraction in ASE.

The extractions were pursued either by Mojonnier or ASE method. The Mojonnier method is an ambient temperature L-L extraction protocol. The solvents used were mixed ethers. The sample prep and extraction was pursued according to AOAC method 996.06.

The ASE is a high-pressure, high-temperature automated solid-liquid (S-L) extraction system. Hexane was used as a solvent for ASE extractions.

### ASE Extraction Conditions

#### Standard Method for Acid-Hydrolyzed Samples

Solvent:	Hexane
Temperature:	100 °C
Pressure:	1500 psi
Heating:	5 min
Static:	5 min
Flush Volume:	30%
Cycle:	3
Purge:	120 s
Total Time:	25 min

#### Standard Method for Base-Hydrolyzed Samples

Solvent:	Hexane
Temperature:	110 °C
Pressure:	1500 psi
Heating:	6 min
Static:	15 min
Flush Volume:	30%
Cycle:	1
Purge:	120 s
Total Time:	25 min

### Parallel Extraction Conditions

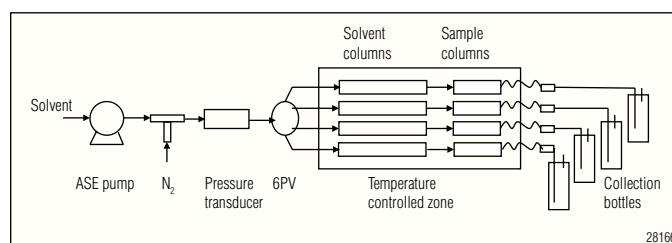


Figure 3. Illustration shows prototype of an instrument setup for parallel extraction.

The sample was prepared by placing the 1:1 sample to ASE Prep DE into a mortar, grinding and mixing by pestle, then loading into stainless steel HPLC column.

### Partial Parallel Extraction (PPE) Conditions

Solvent:	Hexane/dichloromethane/methanol (5:2:1)
Temperature:	100 °C
Static extraction time:	30 min
Purge:	4 min

## RESULTS AND DISCUSSION

The following tables compare the performance of the ASE extraction method with the Mojonnier method. Several food samples were extracted and analyzed. The recovery is reported here as a percentage of the label claim. Excellent recoveries were observed for all the samples with good reproducibility. The ASE method results also compared well with the Mojonnier method. Overall the ASE method was easier to implement and enjoyed the advantage of automation and speed versus the Mojonnier method. In contrast, the Mojonnier method was labor intensive and involved a multistep process.

**Table 1. Gravimetric Results for Lipid Recovery (n = 3) in Food Samples (Acid-Hydrolyzed) for the ASE Resin Method and the Mojonnier Method**

Food		ASE Resin Method Lipid Recovery (%)	Mojonnier Method Lipid Recovery (%)
Corn Chips	AVG	99.52	98.95
	RSD	0.66	0.65
Mayonnaise	AVG	100.10	99.99
	RSD	0.55	0.88
Parmesan Cheese	AVG	96.97	97.98
	RSD	0.80	0.98
Bologna	AVG	99.05	100.70
	RSD	1.30	1.00

**Table 2. GC/MS Results for Lipid Recovery (n = 3) in Food Samples (Acid-Hydrolyzed) for the ASE Resin Method and the Mojonnier Method**

Food		ASE Resin Method Lipid Recovery (%)	Mojonnier Method Lipid Recovery (%)
Mayonnaise	AVG	96.53	97.65
	RSD	0.58	1.20
Bologna	AVG	100.10	100.00
	RSD	1.30	0.97

### Addition of Ethanol after Base Hydrolysis

The base hydrolysis method (AOAC 996.06) recommends mixing samples with 2 mL ethanol, 4 mL water, and 2 mL of 58% NH<sub>4</sub>OH (28–30% NH<sub>3</sub> content); and heating at 70–80 °C for 10–20 min with moderate agitation for hydrolysis.

The ASE extraction of base-hydrolyzed milk samples showed poor lipid recoveries when hexane was the solvent and with addition of 2 mL of ethanol as per AOAC protocol. To address the poor recovery, experiments to study the effect of ethanol on extraction were pursued.

The addition of ethanol to hexane increases the polarity of the solvent. Repeating the extraction method with increasing amounts of ethanol added in addition to the recommended 2 mL improved the recovery as evident from Table 3. The added ethanol imparts some polarity and aids an improved extraction of the fat, possibly by disrupting the stabilizing proteins around the fat globules. It is evident from Table 3 that good recovery is achieved somewhere in the 3 to 4 mL regime.

To ensure good recovery under a variety of sample conditions, the amount of ethanol was increased to 6 mL. For ASE extraction based on this work, it is recommended to add 6 mL of ethanol to the base hydrolyzed sample vial after hydrolysis and before ASE cell prep. Studies pursued with and without ethanol added for a variety of samples demonstrated that this addition was only needed with milk samples.

**Table 3. Gravimetric Results for Lipid Recovery (%) in Milk Sample for the ASE Resin Method\* and the Mojonnier Method**

Sample	ASE, Resin Method Lipid Recovery (%)	Mojonnier extraction Lipid Recovery (%)
Milk (without added ethanol)	48	105
Milk (with 2 mL added ethanol)	100	105
Milk (with 6 mL added ethanol)	103	105

\*With and without adding ethanol after base hydrolysis

**Table 4. Gravimetric Results for Lipid Recovery (n = 3) in Food Samples (Base-Hydrolyzed) for the ASE Resin Method and the Mojonnier Method**

Food		ASE Resin Method Lipid Recovery (%)	Mojonnier Method Lipid Recovery (%)
Heavy Whipping Cream	AVG	101.20	100.20
	RSD	0.16	0.11
Half & Half	AVG	106.40	106.30
	RSD	0.51	0.92
Milk	AVG	103.00	104.80
	RSD	0.16	0.64
Sour Cream	AVG	97.58	94.22
	RSD	0.88	1.30
Cream Cheese	AVG	97.99	98.33
	RSD	1.60	0.66
Condensed Milk	AVG	106.80	107.40
	RSD	0.52	0.84

**Table 5. GC/MS Results for Lipid Recovery (n = 3) in Food Samples (Base-Hydrolyzed) for the ASE Resin Method**

Food		ASE Resin Method Lipid Recovery (%)
Heavy Whipping Cream	AVG	99.20
	RSD	1.80
Half & Half	AVG	102.80
	RSD	1.40
Milk	AVG	98.40
	RSD	0.65
Sour Cream	AVG	96.20
	RSD	2.90
Cream Cheese	AVG	98.10
	RSD	1.70

## ASE OPERATION

### Solvent Usage and Solvent Introduction

- Fill with solvent to set pressure at 1500 psi (or set pressure for ASE 200).
- When the cell heats up, the pressure increases and the static valve opens at 1700 psi (pulse), releasing the pressure.
- Fresh solvent will be pumped in to attain 1500 psi.
- Initiate a flush (as a percentage of the cell volume) at the end of a cycle.
- Multiple cycles may be repeated.

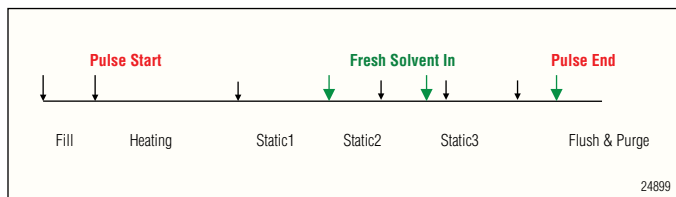


Figure 4. Solvent introduced during fill, pulse, and flush.

## New Mode—Solvent Saver (SS) Mode

### SS Pressure Mode—ASE 350 Accelerated Solvent Extractor

- Fill cell with solvent to set pressure of 1500 psi.
- Turn pump off.
- Hold for prescribed time (static heating). If pressure exceeds 1700 psi, static valve will release pressure but no additional solvent will be pumped in.
- Multiple cycles are not needed.
- Static valve opens and purge occurs after hold time.

### SS Flow Mode—ASE 350 Accelerated Solvent Extractor

- Fill cell with solvent to set pressure of 1500 psi.
- Hold for prescribed heating time. If pressure exceeds 1700 psi during heating and static time, static valve will release pressure and additional solvent will be pumped in.
- Multiple cycles are not needed.
- Extraction continues as pump meters in user-specified aliquots of solvent per minute with regular release of excessive pressure from the cell by opening the static valve.
- Typical solvent flow rate: from 0.5 mL/min to 5 mL/min for various cells.
- Purge occurs after extraction time.

### SS Mode Advantages

- One-half to one-fifth less solvent is consumed compared to existing standard mode of operation. In addition, post-extraction solvent evaporation time is minimized due to lower solvent usage.
- Temperature profile is easier to maintain because influx of solvent is minimized.
- Analysis time can be shortened.

**Table 6. Comparison of Solvent Usage by the Standard Mode Versus the SS Pressure Mode**

Instrument	Sample	Cell Volume	Standard Mode (mL)	SS Pressure Mode (mL)
ASE 150	Infant Formula App. No. 329	10	30	<b>8</b>
ASE 350	Infant Formula App. No. 329	34	81	<b>22</b>
ASE 350 Resin Method	Mayonnaise Acid-Hydrolysis	100	100–110	<b>52</b>
ASE 350 Resin Method	Bologna Acid-Drain	66	79	<b>31</b>
ASE 350 Resin Method	Milk Base-Hydrolysis	100	110–115	<b>65</b>

**Table 7. Gravimetric Results for Lipid Content (n=3) in Acid Hydrolyzed Food Sample for the ASE Resin Method (Various Modes) and the Mojonnier Method**

Food	ASE Standard Mode Lipid Recovery (%)		ASE SS Pressure Mode Lipid Recovery (%)
	AVG	100.10	99.96
	RSD	0.55	0.49
Mayonnaise	ASE SS Flow Mode Lipid Recovery (%)		Mojonnier Method Lipid Recovery (%)
	AVG	100.30	99.99
	RSD	0.37	0.88

**Table 8. Gravimetric Results for Lipid Recovery in Base Hydrolyzed Food Samples for the ASE Resin Method (Various Modes) and the Mojonnier Method**

Food	ASE Standard Mode Lipid Recovery (%)	ASE SS Pressure Mode Lipid Recovery (%)
Whipping Cream	101	99
Half and Half	107	107
Milk	103	102
	ASE, SS Flow Mode Lipid Recovery (%)	Mojonnier Extraction Lipid Recovery (%)
Whipping Cream	100	100
Half and Half	107	106
Milk	103	105

## Partial Parallel Extraction (PPE) Mode

In the PPE mode, multiple cells were extracted simultaneously. Current investigations focused on a format in which columns were filled and purged sequentially (flow was not split), but extractions were pursued in parallel. The cells were filled with solvent for a set time by using an ASE pump. A six-position valve was used to switch the flow to the appropriate cell. The solvent pump was turned off after filling all the cells. The cells were held for the prescribed time (similar to the SS pressure mode). The extracts flowed out of the cell through a restrictor tube to the collection bottle during filling, holding, and purging. A nitrogen purge after the end of the hold time was used to displace all solvent from the cell.

The PPE mode is a high throughput extraction protocol. Extraction time is one-third to one-quarter that of standard extraction. It also lowers the solvent consumption similar to the SS mode.

Excellent lipid recoveries were obtained with two different samples in a four-cell configuration using PPE.

## CONCLUSIONS

The Mojonnier method is a labor intensive and time consuming extraction method. It also exposes the practitioner to concentrated acid and solvent when manually implemented. The phase separation process is subjective to human errors.

In comparison, the ASE method is a fully automated extraction technique where labor savings offset the capital costs. The new resin method for acidic or basic sample matrices allowed in-cell sample neutralization and extraction. The ASE method provides excellent lipid recovery from acid or base hydrolyzed food samples and creates opportunities for other applications that use harsh samples or reagents. The SS modes minimize the usage of solvent. The SS modes improves temperature performance of the cell. It also lowers the solvent usage and evaporation time, reducing the cost of analysis per sample. The new PPE mode is a high-throughput extraction mode which saves labor, solvent and time.

**Table 9. Gravimetric Results for Lipid Recovery in SRM 1849 Infant/Adult Nutritional Formula and Infant Formula Samples in PPE**

Sample	Standard ASE Recovery (%)	Cell 1 Recovery (%)	Cell 2 Recovery (%)	Cell 3 Recovery (%)	Cell 4 Recovery (%)
SRM 1849	101.2	100.3	101.1	100.3	100.7
Infant Formula	101.5	100.8	101.4	100.9	101.3
Extraction Time	140 min 210 min	48 min (4 cells) 57 min (6 cells)			

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LPN 2749-01 3/11  
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