# Microwave-Assisted Amino Acid Hydrolysis of Cheese

## Introduction

Cheese ripening involves several biochemical changes, such as fat hydrolysis, lactose fermentation, and protein degradation. These processes greatly influence the texture, taste, and aroma of the cheese. More specifically, amino acid content and identity affects cheese flavor throughout the aging process. Amino acids in food are both free unbound molecules as well as amino acid residues bound together via peptide bonds, creating the various proteins present in dairy products. In order to fully evaluate the amino acid content of cheese throughout the ripening process, an accurate evaluation of the amino acid composition is necessary. To do this, proteins must be hydrolyzed, effectively liberating amino acid residues, which can then be quantitatively detected in the hydrolysate.

Traditionally, proteins are chemically hydrolyzed under acidic or alkaline conditions upon treatment with 6 N HCl or 4 N NaOH, respectively, in sealed vessels at 110 °C for 18 to 24 hours.[1,2] Factors such as time, temperature, and reagents greatly impact the accuracy and precision of the hydrolysis reaction and thus, the amino acid quantification. Ultimately, amino acid analysis is an expensive, tricky, and time-consuming laboratory procedure. Often, the hydrolysis reaction represents the rate-limiting step for this process. One approach to reduce the reaction time of an organic reaction is to replace conventional heating with microwave energy. Protein hydrolysis reactions can be successfully promoted with microwaves at higher temperatures and in shorter reaction times, compared to conventional heating methods.[3] The Discover® 2.0 microwave reactor from CEM can provide laboratories with the tools to hydrolyze food samples in an efficient, reproducible, and safe manner.

## Materials and Methods

### Reagents and Samples

All reagents were purchased from commercial suppliers and hydrolysis solutions were freshly prepared prior to use. Cheese samples were gathered from a local grocery, homogenized in a food processor, and stored at 4 °C until analysis. An analytical balance was used to aliquot 300 mg portions of each sample for its designated hydrolysis reaction. The amino acid content of the hydrolysates was determined via pre-column derivatization followed by UPLC injection and PDA detection at 260 nm. The Waters AccQ-Tag™ Ultra Derivatization kit was used for LC-PDA analysis of the hydrolysates. All samples were analyzed in triplicate.

### Traditional Acid Hydrolysis Method

A glass vial was charged with a 300 mg portion of sample followed by 2.5 mL of 6 N HCl containing 3% (w/v) phenol solution. The reaction vial was then purged with $N_2$ and quickly sealed. This procedure was repeated until all samples were ready to be placed in a 110 °C oven for 20 hours. Upon completion, samples were removed from the oven and allowed to cool to room temperature prior to derivatization.

### Discover 2.0 Hydrolysis Method

Reaction Set-Up: A 300 mg sample portion was added to a 35-mL Pyrex vessel equipped with a stir bar. A volume of 5 mL of 6 N HCl w/ 3% phenol was then added to each microwave reaction vessel.

Vessels were purged with $N_2$ for 5 minutes, quickly sealed with a Teflon® lined silicon cap, and placed in the autosampler rack for automated placement in the Discover 2.0 cavity.

Method Programming: A one-step Dynamic method was programed in the Discover 2.0 system for the amino acid hydrolysis of cheese.

The following method parameters were used:

**Acid Hydrolysis**
Vessel Type: Pyrex
Control Type: Dynamic
Temperature: 165 °C
Time: 20-min
Pressure: 300 PSI
Power: 300 W
Stirring: High

## Sample Preparation for Analysis

All hydrolysate solutions were passed through 0.2 µm PTFE filters and neutralized with 6 M NaOH in preparation for AccQ-Tag derivatization. A volume of 70 µL of borate buffer from the Waters AccQ-Tag Ultra Derivatization kit was added to a fresh recovery vial. 10 µL of the neutralized sample was added, and the vial was capped and vortexed. Then, 20 µL of prepared derivatization reagent from the kit was added to each sample reaction. The reaction was vortexed for 10 seconds and then was heated at 55 °C for 10 minutes prior to analysis.

## Analysis

A volume of 5 µL of each derivatized reaction was injected into a Waters Acquity H-Class UPLC hooked to a Waters PDA detector. Separation was done with a Waters AccQ-Tag Ultra C18 column (1.7 µm, 2.1 x 100 mm) using a flow rate of 0.25 mL/min. The column temperature was 45 °C. The elution gradient is shown in **Table 1**. The mobile phases were A: Waters AccQ-Tag Eluent A diluted 10-fold in MilliQ water and B: Waters AccQ-Tag Eluent B. To establish amino acid concentrations for each amino acid, Waters Amino Acid Standard (Waters Corporation, Part No. WAT088122) was derivatized at concentrations 5, 10, 25, 50, and 100 pmol/µL.

**Table 1**. Gradient used for Derivatized Amino Acid Separation

| Time (min) | Flow (mL/min) | %A | %B |
|---|---|---|---|
| Initial | 0.250 | 99.9 | 0.1 |
| 0.50 | 0.250 | 99.9 | 0.1 |
| 28.00 | 0.250 | 90.0 | 10.0 |
| 32.00 | 0.250 | 75.0 | 25.0 |
| 34.00 | 0.250 | 0.0 | 100.0 |
| 36.00 | 0.250 | 0.0 | 100.0 |
| 36.01 | 0.250 | 99.9 | 0.1 |
| 40.00 | 0.250 | 99.9 | 0.1 |

# Results and Discussion

The Discover 2.0 was evaluated to facilitate the acid-assisted amino acid hydrolysis of cheese. Results were compared to the traditional oven hydrolysis method performed on the same samples. **Table 2** highlights the amino acid recoveries of cheddar and Colby cheese samples under traditional hydrolysis oven conditions, as well as the Discover 2.0 acid hydrolysis reaction conditions. All of the Discover 2.0 assisted hydrolysis reactions compared well in terms of recovery to their traditional hydrolysis counterparts.

Microwave heating provides rapid, efficient, and reliable volumetric heating. This dielectric heating mechanism allows for the transfer of heat to the reaction contents in a uniform and instantaneous manner.[4] Conventional heating methods, such as ovens and heating mantles, rely on the transfer of heat through the vessel walls, then through the reaction components, which results in slow and inefficient heating as well as undesired temperature gradients and side reactions. Also, the addition of stirring during the Discover 2.0 hydrolysis reactions results in increased reaction homogeneity. Lastly, compressed air rapidly cools the microwave-assisted reactions to a safe-handling temperature, allowing for decreased time to sample analysis. Ultimately, the Discover 2.0 offers an alternative route to amino acid hydrolysis with key benefits, including shorter reaction times, cleaner hydrolysates, and quicker time to analysis.

**Table 2**. Percent Recoveries for 15 Amino Acids in Cheese

| Amino Acid | Cheddar | Colby |
|---|---|---|
| Asp + Asn | 130.95% | 92.12% |
| Ser | 103.51% | 131.51% |
| Glu + Gln | 130.45% | 92.37% |
| Pro | 121.72% | 116.10% |
| Gly | 106.10% | 106.72% |
| Ala | 114.38% | 112.27% |
| Val | 113.87% | 108.77% |
| Ile | 114.35% | 114.09% |
| Leu | 105.31% | 105.66% |
| Tyr | 92.63% | 75.48% |
| Phe | 103.65% | 106.96% |
| Lys | 126.55% | 101.21% |
| His | 107.47% | 100.98% |

## Conclusions

Acid hydrolysis followed by derivatization and characterization of the resulting amino acids was performed on two different cheese samples to determine the complete amino acid profile. Comparison of a traditional thermal method to a rapid microwave method demonstrated comparable or, in most cases, better recoveries of the amino acids with a substantial reduction in reaction time. These results indicate the Discover 2.0 system can be used as a faster, safer alternative to monitor cheese throughout the production process, providing insight into ripening, flavor profile, and more.

## References

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**United States (Headquarters)**
800-726-3331
704-821-7015
info@cem.com

**France**
33 (01) 69 35 57 80
info.fr@cem.com

**Germany, Austria, Switzerland**
(49) 2842-9644-0
info@cem.de

**Ireland**
+353 (0) 1 885 1752
info.ireland@cem.com

**Italy**
(39) 35-896224
info.srl@cem.com

**Japan**
+81-3-5793-8542
info@cemjapan.co.jp

**United Kingdom**
(44) 1280-822873
info.uk@cem.com

www.cem.com