IUN (Interesterase Units Novo) Activity Determination by Direct Measure NMR of Solid Fat Content at 40°c

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Principle

The method is based on the the ability of the immobilized enzyme to alter the fat compostion in a mechanically rotated, thermally controlled oil system via intermolecular rearrangement (interesterification). The Brukker minispec20 nmr configured for Solid Fat Content (SFC) has the ability to differentiate solid/liquid fat proton environments and can quantitatively report a ratio of these as a percentage. Exposure to the immobilized enzyme over a contsant time will convert the oil to a lower solids value depentant on the potency of the enzyme. The rate at which the SFC changes over at constant time is measured and related back to a tristearin value. The tristearin value is the definition of the IUN. An initial SFC measurement is made on the nontreated oil and compared to a SFC value made after 30 minute exposure to the enzyme. Assay reproduciblity is achieved using tempering protocols derrived from A.O.A.C proceedures for controlled solidifaction, direct measurement SFC at 40C.

Unit Definitions

1 IUN = 0.01 % Tristearin decrease/minute/gm enzyme in a RBDO/ FHSO mixture.

A reference batch LA350008 (320 IUN/gm) was used to determine a %SFC / %Tristearin factor as such.

1% SFC/min = 12.6 % Tristearin/min under conditions of this procedure.

NOTE: This is only applicable to this procedure using this oil system and conditions.

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Reaction Conditions

- Temperature: 70°C ± 0.05°C
- Reaction Time: 30 minutes
- Mobilization Mode/Velocity: In situ rotational shaking at 200 rpm
- Tempering Flow: 100°C ± 2°C for at least 15 min. or up to 30 min.
  60°C ± 0.1°C for 5 min. ± 1 min.
  0°C ± 0.1°C for 60 min. ± 1 min.
  40°C ± 0.1°C for 30 min.
  Measure to instrument at 40°C

Specificity and Selectivity

Lower Limit of detection: 50 IUN/gm as the most significant different measurable SFC relative to the background SFC measurement. Maximum Limit of detection: 600 IUN/gm as the lowest significant and reproducible SFC measurement obtained under current calibration procedure (See Note1). It is of high importance that the enzyme solids are representative between duplicates and do not contain high moisture contents. Validation data demonstrates significant differences in IUN values between particle sizes. Spin riffling devices are recommended for subsampling and for generating duplicate test fractions. Hydrolysis reactions interfere with interesterification. Samples with high moisture (> 5 %) should be dried prior to analysis. Very small particle sizes (< 100 microns) will disperse/suspend in oil. The measurement is to be made on an oil fraction which is devoid of enzyme with is easily settled out. Assay is specific for this oil system only and under these tempering conditions. Its is recommended to stay within the constraints of 1) enzyme weights 2) substrate weights) 3)glassware and 4) and rotating apparatuses.

NOTE: The Brukker mq20 is calibrated using a 0.0%, 31.1%, and a 71.1 % solids content standard. These are Brukker certified standard tubes and are used as a primary standard once daily. Method currently has seen no values below 5% SFC, therefore reproducibility may not be guaranteed below this.

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Equipment

Quantachrom Spinning Riffler Model RR-2 or equivalent

Reaction bath: Boekel Grant ORS 200. Temp at 70.0C (+/-0.1C) Rotation at 200 rpm.

125 ml Screw Top Erlenmeyer flask for Reaction Mixture.

Tempering Bath 100C (+/-2C) Pierce Reacti-Therm Dry Block Heater.

Tempering Bath 60C (+/-0.1C) Julabo MC 17.

Tempering Bath 0C (+/-0.1C) Julabo F 34 using 15% Glycol as medium.

Tempering Bath 40C (+/-0.1C) Julabo MC 17.

Brukker minispec mq20 v2.51 Rev 00/NT

MqFirmware v2.50 Rev 00

TempControl Firmware v1.90 (Magnet at 40C)

NOTE: Each bath is equipped with Standard aluminium 20 NMR tube heating blocks. (Height=70 mm,Depth = 75mm, W =100mm) All bath temperatures set to specs using Testo 454 Thermal reference probe (or equivalent) in situ.

Substrate

Substrate

A: Refined Bleached Deodorized Soy Oil RBDSO (from ADM)

B:Fully hydrogenated Soy Oil FHSO(from ADM).

RBDSO and FHSO are mixed 73:27 wt/wt respectively. Solution is placed in 70C oven until melted and homogeneous. Once melted and homogeneous 20 gm aliquots are placed in screw top 125 erlenmayer flasks. The flask may be kept one week at room temperature before analysis. SFC 40 Should be at 20.7 % +/- 0.5.

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Tempering Controls

Glyceryl Tripalmitin Sigma T8127 C51H98O6 98%

Glyceral Triolein Sigma T7752 C57H104O6 Practical Grade 65%

Standard #	Grams of Tripalmitin	Grams of Triolein
1	1	24
2	5	20
3	10	15

NOTE: Solutions are melted at 70C, then aliqouted into NMR tubes and labelled. Reuse of these tubes is acceptable.

Samples and Controls

Samples are split to ca 1 gm levels. It is recommended to use between 0.8 and 1.2 gm for each test to keep enzyme oil ratio constant. Splitting and shuffling splits may be necessary to deliver these targets weight. Batch LA350008 uses a declared activity of 320 IUN/gm and is avaiable from reference lab(NZNA)

One tempering control is analyzed per Run (see procedure) and data is monitored for this. A tempering control set is available from the reference lab with a current value and limits. A set can also be prepared as mentioned above for day to day monitoring.

Run Definition

A maximum of 20 tubes are processed per 1 tempering.

1 tempering includes: Assay control first duplicate [1 tube]
Samples run (in duplicate) max are 8 samples [16 tubes].
Assay control second duplicates [1 tube].
Blank tube (Background) [1 tube].
Tempering control. Can be any 1 of the set [1 tube].

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Sample Preparation

Follow the steps below to prepare the samples for IUN:

Step	Action
1	Weigh 20 ± 0.05 grams of melted aliquots of RBDSO/FHSO into 125 mL screw top Erlenmeyer flask.
2	Aliquot a portion of the melted oil into 1 nmr tube and keep at room temperature until assay solutions are run. *NOTE:* This is a blank (T0 value)
3	Melt and re-equilibrate all 125 mL flask used in one run in the 70°C bath while rotating at 200 rpm in the ORS 200 bath.
4	Place all of the enzyme samples (ca 1 gram of known weight) to be tested into a test tube rack or any other container so that they can be delivered quickly to the 70°C equilibrated oils.

Assay Procedure

Follow the steps below to perform the assay:

Step	Action
1	Start timer and deliver Enzyme test samples into the 20 gm oil fraction. *NOTE:* Rotation in water bath is halted. A stainless steel funnel is used to delivered sample into the flask at time zero. After the sample has been added the rotation is switched back on. One minute intervals between samples are a comfortable working arrangement.
2	Terminate the reaction after 30 minutes by switching off the rotation and removing the flask from the bath.
3	Tilt enzyme/oil mixture at a 45 degree angle to settle the granulate.

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Assay Procedure *(continued)*

Step	Action
4	Transfer a ca. 2.5 ml portion of oil to the nmr tube. *NOTE:* NMR Tube Filling: The constraints are as follows: Minimum of 2 cm from bottom of tube for measurement. Maximum of 1 cm below tempering block for thermal containment. A reference tube marked at 2 and 5 cm will provide as a good guideline for tube filling. It is not intended to have a quantitative aliquot delivered from flask to tube. 2 cm is the height of the coil in the NMR and 5 cm will keep the aliquot thermally optimized during the tempering process.
5	Place the nmr tube into the 100°C heating block. *NOTE:* Once the reaction is over, the flasks are turned upside down into some sort of containment vessel so the fat will drain. This will assist in glassware cleaning. Soak the used glassware in 10% Micro 90. This solution will remove fat stuck on glassware.
6	Repeat steps 4 and 5 until all tubes have been placed in the 100°C heating block.
7	Place the blank and tempering control in the 100°C heating block after the last flask has been drawn and completed.
8	Hold all tubes at this temperature for 15 minutes.
9	Transfer all tubes to 60°C heating block and hold for 5 minutes.
10	Transfer all tubes to 0°C block and hold for 60 minutes.
11	Transfer all tubes to 40°C block and hold for 30 minutes.
12	Read and record SFC at 40°C in minispec.

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Calculations

Blank (or background) %SFC = SFC40Blank

%SFC after 30 minutes of rotating at 200 rpm 70C = SFC40T30

[SFC40Blank – SFC40T30] / 30 min = % SFC/min

% SFC/min * 12.6 = % Tristearin / min

[% Tristearin / min] / 0.01 = IUN

IUN / SamWt(gm) = IUN/g

Example:

1.04 gm Lipozyme TL IM assayed.

SFC40 Blank: 20.5

SFC40 T30 Readings: 10.7

[20.5 – 10.7] / 30 = 0.326 % SFC/min

0.326 * 12.6 = 4.12 % Tristearin/ min

4.12 / 0.01 = 412 IUN

412 / 1.04 gm = 395 IUN/g

Repeat for duplicate within run. Main Batch analysis should require 2 runs for average value.

Acceptance Criteria for Approval of a Run

Checks and cross references. Primary calibrators must meet instrument criteria as supplied in software. This cross references AOAC procedures(See referennce 1).

Blank Value should be reproducible at 20.6 +/- 0.5 (mean +/- 3s).

Tempering standards to control thermal movement should be reproducible to 2% of Declared (or determined) SFC.

Daily control of reaction can be maintained though repetitive use of in house material if desired or reference material can be supplied from NZNA.

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Statement of Analysis Results

Minimum activity (Detection Limit) is a 1 % SFC change. This is required to meet statistically significant rate data. This would yield a minimum activity of 50 IUN/g.

Assay data of samples between 100 and 600 IUN have been shown to be 5 % reproducible.

The maximum activity is a final T30 SFC measurement above 5 %. This has not been observed so far and is not considered to adhere to the 5 % reproducibility criterion.