Dit project is mede met Europese middelen bekostigd

"Europees Visserijfonds, Investering in Duurzame Visserij"

Samenvatting Valvis Project met

Overzicht van de Wetenschappelijke Bijdrage van de betrokken Kennisinstellingen

Opgeleverd door Marnix ten Kortenaar (penvoerder)

CEO Dr Ten BV

Mede tot stand gekomen dankzij

1) projectpartner Aquamar BV te Urk (Lub Kramer, Dubbele Janson, Marretje Kofferman)
2) Dr Ten medewerkers (Ir Klary Bolt, Gerrit Miedema, Margriet ten Kortenaar, Ir Diego Quintero en Ing. Ilse Pierik):

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Bijdrage WUR-TNO aan Valvis project gedraaid in 2014/2015 onder leiding van Dr Ten BV

1. Samenvatting van het Project (door Marnix ten Kortenaar)

De Nederlandse visvangst gaat gepaard met het terugwerpen van vele duizenden tonnen ondermaatse vis. Daarbij sterft een groter deel van de vis waardoor vele tonnen aan voedingswaarde verloren gaat. Door de komst van nieuwe Europese wetgeving dient de ondermaatse vis op relatief korte termijn in toenemende mate aan wal te worden gebracht en ontstaan nog grotere hoeveelheden visafval. Visafval heeft economisch gezien voor Nederland op dit moment relatief weinig waarde omdat in o.a. Noorwegen en Denemarken de verwerking tot relatief laagwaardig vismeel plaatsvindt (< €1,00 per kg). Een tot op heden gemiste kans voor o.a. Urk omdat nog niet kon worden gekomen tot grootschalige, duurzame en slimme valorisatie van het afval tot producten met meer economische waarde.

In de afgelopen jaren is door Dr Ten BV en partners mede in opdracht van het Ministerie van Economische zaken onderzoek verricht naar de biologische omzetting van melkvet tot gezonde lipiden ter verwerking in o.a. creamer, kindervoeding, zuivel- en sportvoeding. Tijdens dit onderzoek werd oriënterend gekeken naar de valorisatie van vis(afval) uit Urk. Het werk legde de basis voor het Valvis project, een project waarbij valorisatie van vis(afval) en ondermaatse vis centraal stond.

Dit project betrof een brede praktische georiënteerde R&D haalbaarheidsstudie om te kijken of kon worden gekomen tot een brede duurzame, biologische valorisatie van het vis en afval (zowel fileerafval als discards) op Urk. Uit allerlei soorten vis(afval) werd olie geïsoleerd waarna allerlei enzymatische omzettingen werden bestudeerd. Daarnaast werden er emulsie en sproei droog proeven gedaan en ook gekeken naar de opschaling van het extractie-, enzym- en poederproces.

Zijdeling werd gekeken of kon worden gekomen tot valorisatie van overtollig glycerol afkomstig van het visafval bij olie extractie en omzetting. Een nieuwe brandstofcel die het glycerol omzet in duurzame energie werd op lab schaal gedemonstreerd. Een klinische oriëntatie werd tevens verricht om te kijken of visolie en andere triglyceriden een basis kunnen vormen voor nieuwe sportvoeding. Ook werd gekeken naar wet- en regelgeving, strategie, samenwerking, sourcing/business development buiten EU (Noorwegen/Israël), partners.

Het onderzoek heeft aan de van hand van praktische experimenten en toetsing geleid tot de conclusie dat vis(afval) een sleutel vormt tot het gezonder en goedkoper maken van zuivel ingrediënten en omgekeerd dat melkvet (room) kan leiden gezonde, goedkope, nieuwe, laag calorische visolie. Enzymen, slimme extractie, encapsulatie en smaak optimalisatie en intelligente implementatie in de markt vormen de hoofdtools om de hobbel te nemen die leidt tot nieuwe technische en economische positionering van Nederlands nationale grondstoffen melk en vis in intelligente producten.

Nederland kampt met toenemende gezondheidsproblemen als gevolg van overgewicht en hart- en vaatziekten. Daarnaast kampt de zuivelindustrie met een ongezond imago door het hoge aantal verzadigde vetzuren in zuivel. Korte vetzuren en meervoudig onverzadigde vetzuren en biologische omzetting vormen een sleutel tot het bestrijden van deze problemen. Daarnaast staan vissers voor het probleem om de grote toename van niet te veilen vis rendabel te maken doordat met de komst van nieuwe wetgeving discards aangeland moeten worden. De meest waardevolle stoffen uit vis waaronder meervoudig onverzadigde vetzuren en eiwitten worden nog te weinig aangewend om de volksgezondheid te dienen.

De combinatie van vis en zuivel plaveien dus de weg tot het gezonder maken van zuivel enerzijds (en daarmee het versterken van de Nederlandse concurrentiepositie van de zuivelsector) terwijl de visserijsector anderzijds een economische boost krijgen doordat verhoogde afzet plaats kan vinden in bijvoorbeeld babyvoeding doordat olie discards kunnen worden opgewerkt tot high value ingrediënten. Afvalstromen kunnen bovendien lokaal worden verwerkt waardoor er minder transport van grondstoffen plaatsvindt wat ten goede komt aan milieuaspecten.

Dit project heeft verder aangetoond dat een lucratieve bio-food industrie kan worden opgebouwd in Urk waaruit werkgelegenheid voortkomt en uiteindelijk de volksgezondheid kan worden gediend door lipiden op de markt te brengen op basis van visolie (omega 3 en 6 vetzuren) en melkvet (kortere vetzuurketens). Dergelijke lipiden hebben een positief effect op hart- en vaatziekten en bijvoorbeeld de hersenontwikkeling bij jonge kinderen. Een nieuw onderzoek met de WUR heeft als doel om ook de vis eiwitten nader op industriële valorisatie te bekijken o.a. tezamen met Holland Diamond Fish, een nieuwe Urkse start up in o.a. nieuwe vissaus onder leiding van Pieter Kapteijn. Daarnaast wordt met een Israëlisch bedrijf nog gekeken of een project kan worden opgezet waarbij vis in aquabaden efficiënter kan worden gekweekt zodat de discard situatie nog beter kan worden beheerst. Als toets gelden kleine Israëlische Petrus visjes uit het meer van Galilea die in Urk tot groei moeten komen. Naar zeggen van de Urkers gelijkt deze koshere vis (vernoemd naar de Joodse Christus apostel van Jeruzalem die worstelde met de kraaiende haan te zien op elke kerk), aan de schellevis in de vlag van Urk…

Wetenschappelijke bijdrage in het project

Het consortium is dank verschuldigd aan het Ministerie van Economische Zaken, de provincie Flevoland en de Europese Unie voor financiële ondersteuning van het project. Daarnaast is er ondersteuning en wetenschappelijk advies geweest vanuit de WUR-imares en de Universiteit Twente. Met de WUR zijn meerdere advies gesprekken gevoerd rond de vis- en discard situatie, hierin met name dank aan Drs Jeroen Kals en Ing. Marnix Poelman maar ook Dr Michael Kotterman. Ook zijn diverse bronnen van de WUR/TNO geraadpleegd (waaronder met name het proefschrift van Isabel Aidos ‘Production of High-Quality Fish Oil from Herring Byproducts’ 2002 alsmede een TNO rapport rond extractive van visolie en het LEI rapport van de WUR rond discards (Buisman et. Al. ‘Effecten van een verbod op discards in de Nederlandse visserij’, Lei rapport 2011-014).

Verder zijn zijn diverse analyses gedaan door imares onder leiding van Dr Kotterman op oriënterende enzymatische hydrolyse reacties. Ook werd onder wetenschappelijke begeleiding van Dr Mensink gekomen tot een korte vergelijkende klinische orientatie met de Isala klinieken waarin werd gekeken wat het verschil was tussen een preparaat met korte vetzuren (tributyrin) en visolie. Tenslotte werd met inmiddels oud— Dr Ten medewerker en promovendus Ir Diego Quintero onder leiding van prof. Dr Gerard Smit van de Universiteit Twente gekeken of een outlet kon worden gevonden voor eventueel overtollig glycerol middels een nieuwe brandstofcel (glycerol is een bijproduct bij enzymatische omzetting van visolie naar bijvoorbeeld ethylesters).

Vanwege de publicatieplicht van publieke kennisinstellingen is Dr Ten gevraagd een overzicht te geven van het wetenschappelijke werk gelieerd aan het project en boven beschreven. Na deze samenvatting volgt daarin derhalve een overzicht.

Achtergrond Dr Ten

Dr Ten (www.drten.nl) is een MKB bedrijf uit Wezep dat bestaat uit ca. 5-8 medewerkers, grotendeels ingenieurs die werken aan de energie, water en food technologie, van de toekomst. Dr Ten is opgericht in 2008 door oud schaatser, wetenschapper Dr Marnix ten Kortenaar. Dr Ten werkt voor het MKB, overheid, multinationals, NGO’s. Het doet projectontwikkeling, onderzoek, engineering en advies. Het heeft een klein eigen lab maar werkt ook met faciliteiten in universiteiten en bedrijven. Eerder werkte oprichter ten Kortenaar voor o.a. DSM, FrieslandCampina, TU Delft. In de afgelopen jaren heeft Dr Ten o.a. onderzoek verricht naar de enzymatische valorisatie van melkvet waarbij zijdelings een haalbaarheidsstudie werd verricht naar de valorisatie van visafval (zie ook RVO OneLip eindrapportage, FND project 2012).Kwalitatief hoogwaardige olie kon worden gewonnen die tevens kon worden geinteresterificeerd tot nieuwe lipiden. Het onderzoek legde de basis voor het huidige project. Dr Ten trad op als penvoerder en coördineerde het project. Daarnaast verrichtte het onderzoek naar de enzymatische omzetting van de visolie alsmede het smaakloos verpoederen en het gebruik in creamers, gezondheid voeding en babyvoeding. Vanuit de goede relatie met grote bedrijven tracht Dr Ten om de nieuwe producten aan te laten sluiten bij productapplicaties van dergelijke bedrijven.

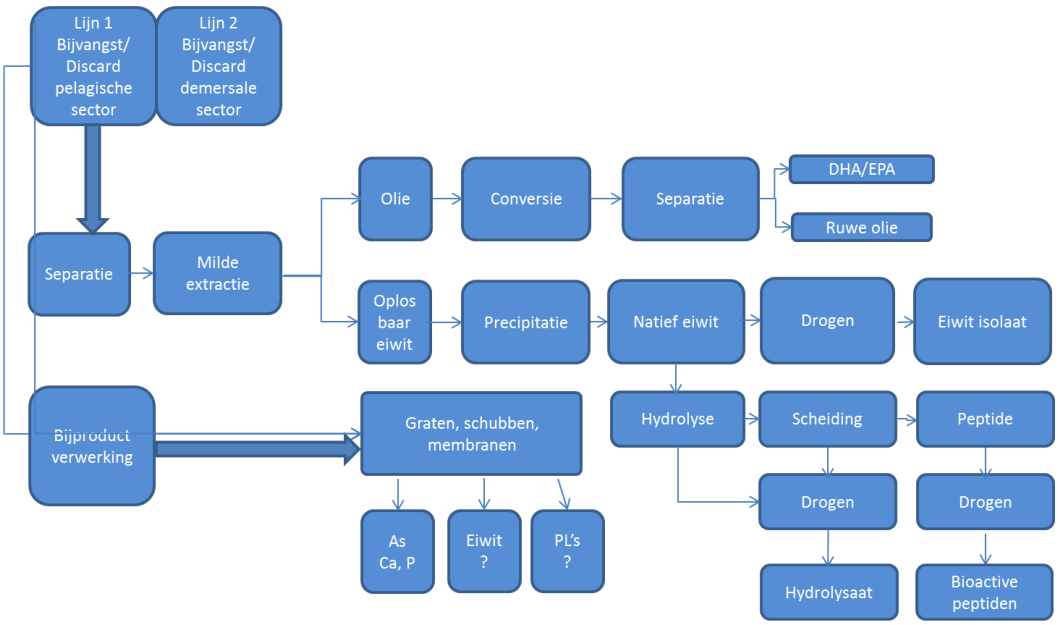
Wetenschappelijke bijdragen

1. Notitie Ing. Marnix Poelman, Drs Jeroen Kals (imares) rond mogelijke verwerking van vis.
2. Klinische oriëntatiestudie tributyrin versus visolie door Pieter ten Kortenaar onder begeleiding van Dr Mensink (WUR) en Ir Klary Bolt.
3. Oriëntatiestudie naar de valorisatie van glycerol in een brandstofcel door Ir Quintero onder begeleiding van Marnix ten Kortenaar en Prof. Dr. Gerard Smit (UT).

1. Notitie Ing. Marnix Poelman, Drs Jeroen Kals (imares) rond mogelijke verwerking van vis.

De Nederlandse demersale visserijsector krijgt, per 1 december 2016 (voorlopige planning) te maken met de discard van als gevolg van de implementatie van het Europese visserij beleid. Dit betekent dat discards, die voorheen teruggegooid werden, dan aangeland moeten worden. Hierdoor ontstaat een nieuwe productstroom, die voorheen niet benut kon worden. De discardban brengt hoge kosten voor het visserijbedrijf met zich mee, waardoor het visproduct in kostprijs toeneemt. Daarnaast is de discard een uitstekende bron voor dierlijke eiwitten en vetten, die niet verloren mag gaan. De meeste gedachten om de discards te verwaarden gaan uit naar bulk productie voor vismeel (dierlijk eiwit en vet), dierlijke bijproduct en biogas productie. Dit beperkt de terugverdien kansen voor de visserij (en hiermee de internationale concurrentiepositie), maar ook de kansen om een waardevolle grondstof op nieuwe wijze met added value in de markt te zetten. Dit project streeft er naar om via het opzetten van nieuwe processen op hoogwaardige wijze gebruik te maken van discards als grondstof voor eiwit en vet producten met toegevoegde waarde. Hierbij gaat het met name om hoogwaardige eiwitten (hydrolysaat en). Dit wordt bereikt door de best practices in bijproduct verwerking te combineren met nieuwe processen en optimalisatie hiervan. Hierdoor wordt een unieke keten ontwikkeld, die kwalitatief hoogwaardige producten ontwikkeld. Het is nodig de haalbaarheid van het proces te identificeren door oa: beoordelen potentie koppeling van de processen, identificeren state-of-the-art, identificatie technische partners, evalueren marktpotentie.

Het proces in de keten:



Risico's

De discards regelgeving is in het leven geroepen om selectieve visserij te bevorderen en de ecologische impact te verminderen. Wanneer de discardproducten een economisch renderende keten vinden kan dit leiden naar een contradictie tussen economische en visserijbeleid belangen. Het project dient er hierdoor voornamelijk voor om de kostprijs van visserij na de discardban te verlagen, door de kosten die voortvloeien uit de discardban te compenseren. Onvoldoende uniciteit van de keten zal leiden tot het produceren van bulk goederen. De kracht van het project is de uniciteit en kwaliteit van de te produceren producten. Hierdoor wordt een niche markt (substantieel) bediend, welke zeer goed aan dient te sluiten bij de concurrerende markten en producten.

2. **Klinische oriëntatiestudie tributyrin versus visolie door Pieter ten Kortenaar onder begeleiding van Dr Mensink (WUR) en Ir Klary Bolt.**

Pieter ten Kortenaar with supervision by Dr Mensink, Ir Klary Bolt
Dr. Ten BV, Wezep

Student number: 921028469010
Course Code: HNE-75324 Nutrition and Health over the Lifecourse
External supervisor: Marco Mensink
Internship Provider: Dr. Ten BV, Rondweg 11 M, 8091 XA Wezep
Internal supervisor: Klary Bolt
Date: Sep-Mar 2015

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Background

Over de course of 2016, my attention was drawn to the biological importance of the so-called short-chain fatty acids (SCFAs). A wealth of scientific literature indicates their potential role in all sorts of diseases, primarily the gastrointestinal ones. Given my background as an elite cyclist - always looking for legal improvements in performance - and bearing in mind the all-ready studied potential of the little longer fatty acids, *mid-chain triglycerides* (MCTs) in enhancing endurance capacity[1], I decided to make some study of the potential of SCFAs as ergogenic aids.

To be clear: scientific proof for MCTs as ergogenic aids is indefinite and ambiguous, and their commercial use is, as far as I can see, restricted mainly to people adhering to a so-called ketogenic diet, mainly because most athletes are not convinced of its benefits over the ingestion of plain carbohydrates in the first place: cheap and proven ergogenics, and palatable ones. MCTs however, are decent precursors to the ketone body *β-hydroxybutyrate* (BHB), and can be of help for those who have difficulties achieving proper plasma ketone levels (>1-2 mM), explaining their use.

Paradoxically, the primary aim of endurance training is to force – and learn - the body to spare glycogen (carbohydrate) at the expense of using the virtually unlimited storage of triglycerides in the adipose tissue. As such, the effects of endurance training rely on the body *super-compensating* to the artificially created state of energy depletion during training, mimicking starvation, or a low-carbohydrate e.g. ketogenic diet.

To my great surprise and satisfaction, butyrate supplementation, a SCFA (C4:0) and close analogue to BHB, has been shown to increase fatty acid oxidation in rats via the key molecular pathways normally targeted by strenuous exercise. The publication covering this topic however, was a mere loner in the vast area comprising studies into the ketogenic diet and MCTs in relation to endurance capacity.

Summary of the publication

Supplementary sodiumbutyrate at 5% wt/wt of a high-fat diet (HFD) given to rats increases fatty acid oxidation (illustrated by the decreased RER and increased EE) and adaptive thermogenesis, whilst gains in body fat are completely diminished[2].

Most strikingly, mitochondrial function and biogenesis in brown fat and skeletal tissue increase as well, as are the levels of type I ('slow twitch') muscle fibers and levels of myoglobin. This finding probably finds its roots in the upregulation PPAR-γ coactivator-1α (**PGC-1α**), whose levels are upregulated at the mRNA and protein level[2].

Levels of **AMPK** and p38 activities are also directly upregulated by C4:0 and may explain the enhancement of PGC-1α activity. Additionally, **CPT-1b**, **PPAR-δ** and cytochrome oxidase I are increased also, whereas plasma triglyceride and cholesterol levels show a decline[2].

The authors of the publication propose that all of the effects of butyric acid are to be attributed to inhibition of histone deacetylase (**HDAC**), thereby upregulating the activity aforementioned genes[2].

As already briefly touched upon, these genes are all involved in the adaptation to endurance exercise, and as such, targeting HDAC with butyrate would theoretically increase endurance capacity, as rationalized by the following quotes:

'...AMPK-PPAR-δ pathway can be targeted by orally active drugs to ***enhance training adaptation or even to increase endurance without exercise*****[3].***'*

'…the group submitted to both exercise mimetics [AMPK and PPAR agonists] *and exercise training presented* ***improved functional performance (…) as well as aerobic capacity*****[4].'**

'...PPARβ/δ agonist and exercise training ***synergistically increase oxidative myofibers and running endurance in adult mice*****[3].'**

*'Mice expressing a constitutively active form of PPARδ were nicknamed '****marathon mice'*** *as they can run for up to twice the distance of their wild-type littermates*[5]*.'*

*'*In vivo *ectopic expression of PGC-1α in skeletal muscle not only induces mitochondrial biogenesis and OXPHOS activity, but also switches type IIb and IIx/d glycolytic fibers to type I and IIa oxidative fibers.* ***As a result, the transgenic mice have improved endurance running performance***[5]***.'***

'Potthoff and colleagues have illustrated ***that*** **over-expression** ***of HDAC5 is*** **negatively** ***correlated with endurance training-mediated adaptations in mouse skeletal muscle*****[6].'**

'...the selective ***degradation of class II HDACs in slow skeletal muscle provides a mechanism for enhancing physical performance and resistance to fatigue*** *by augmenting the transcriptional activity of MEF2*[7]*.'*

Of the SCFAs, butyric acid is the only one with physiologically relevant HDAC-inhibiting properties, but decent proof for my initial idea – that the shorter the fatty acid, the easier it gets oxidized and adds to ATP generation - has been recently confirmed too: *Acetic acid enhances endurance capacity of exercise-trained mice by increasing skeletal muscle oxidative properties,* adding further back-up to my thoughts[8]. To my surprise '*acetic acid significantly increased the muscle expression of key enzymes involved in fatty acid oxidation and glycolytic-to-oxidative fiber-type transformation. Taken together, these findings suggest that acetic acid improves endurance exercise capacity by promoting muscle oxidative properties, in part through the AMPK-mediated fatty acid oxidation and provide an important basis for the application of acetic acid as a major component of novel ergogenic aids.'* Apparently, either acetic acid is a HDAC-inhibitor like butyric acid too, or even other mechanisms are involved in the effects of butyrate[9]. Supposedly, acetic acid alters hepatic acetyl-CoA levels, indirectly modifying the AMP:ATP-ratio[8].

Initiating an internship

So far, so good, but the notion that butyrate actually would bring about improvements in exercise performance still remained speculative and was based on inductive reasoning solely. Presenting the idea to a few members of the Human Nutrition chairgroup engendered some enthusiastic reactions, although this did not result in further actions taken necessary for testing the hypothesis, mainly for organizational and financial reasons. I was advised however, to find a company were I could do a study within the broader framework of an internship.

This led me to contact Dr. Ten BV, a Dutch company located in Wezep near Zwolle, involved in sustainable energy production, water cleansing devices and development of nutraceuticals and food supplements. I knew they had been performing human trials in collaboration with Isala Zwolle, a hospital, in search for new clinically relevant dairy, plant and fish-based biolipids. Perhaps an internship would allow me to answer the questions I had, as well as giving me a clear insight in the preparation of a clinical study, development of novel food ingredients and potential ergogenics.

Besides preparing me for a master thesis, the experience of working in an innovative company – something I admire – looked like a worthwhile investment.

Dr. Ten was as impressed by the studies I presented them and agreed on initiating an internship, starting 1 September 2015 for the duration of 4 months, a total of 24 ECTS. Already before this date I wrote a study proposal for the execution of a human trial at Isala, to be performed during the internship - along with the other activities at the Dr. Ten subdivision Food.

Start of the internship

In the first few weeks at Dr. Ten, my supervisor introduced me to her daily activities, which, at that time, mainly comprised the involvement in the so-called ValVis-project.
The Valvisproject is a Dutch governmental initiative intended to find a solution and worthwhile application for waste and discards of the fishery at Urk. As from next year, 'Brussel' has illegalized the ridding of fish waste in the North Sea, because it enfavours imbalances in the fish stock. Dr. Ten was given funds to find solutions for this problem e.g. to look for possible applications for these discards. Finding new strategies to start-up a processing plant was one of the prerequisites for the project, as was the application of the oil extract in potential commercial products, such as pet food, infant formulas and sports nutrition. In the next section a practical report is presented, just to give the reader some impression of the daily activities during the internship.

Detailed overview of activities

Here, a detailed overview of my activities during the last 2016 trimester is presented. All these activities were somehow related to the ValVis-project, or the butyric acid intervention.

Extraction fish-oil from plaice (Dutch: 'schol')

Goal: To obtain fish oil from approximately 20 kilograms of plaice - for analysis.

Materials: Deep-frozen plaice was provided by a big Urkish fish-handling company, just to give us an idea on how to apply a refining method to waste and discards in the near future.
Furthermore: various stainless steel pans, gas cooker, knives to cut the fish, a press, cans to collect the pressed liquids, a separating funnel etc.

Plaice

Methods: One portion of the fish was treated with an antibacterial substance beforehand. This portion clearly was a little bit whitish in colour, as compared to the other portion, of which the colour tended towards yellow. After thawing the fish, it was cooked in water for 30 minutes, whilst it was cut in small pieces (twice). See picture:

Cooking the plaice

Keeping the pulp warm was key to ensure full extraction of the oil. The hot mixture (soup/broth-like) was then put through a filter placed inside a press. The liquid phase was drained off in glasswork. After a few minutes, a two-layered yellowish liquid was formed with the oil on top. The remaining solids then were pressed, to further egress some oil.

The liquid, non-oily part was then discharged gradually. The remainder of the mixture was fractionated in a more precise measuring cup, so that an (almost) pure oil now was obtained from the fish. At all times, a high temperature was ensured.

Results: The amount of oil extracted from the fish was rather low. First, the fish was of the non-fatty type, and second: our methods leave room for improvement. A specimen of the oil was then subjected to thorough analysis, the results of which can be found in addendum 1.

Draining off the liquid

Separation oily and aqueous layer

Pressing the remaining liquids

Remainder of the fish after pressing

Obtaining a proper source of butyrate for administration

At the same time, we were vigorously ponding on how to find methods for the trial concerning butyric acid. Butyric acid is available in various forms:

1. As a water-like liquid with a strong vinegar taste and pungent smell
2. After ethanolisation, as ethylbutyrate – a food flavouring ingredient with a strong ethanol-like taste and pineapple/grapefruit smell.
3. Upon treatment with sodiumhydroxide as sodiumbutyrate salt, a substance with a soapy, salty, oily taste.
4. Esterified, as the triglyceride/oil of butyric acid: tributyrin.

We decide to order the latter one: tributyrin, obtained at Sigma-Aldrich. There were a few reasons underlying this decision. First: tributyrin is not volatile like butyric acid and ethylbutyrate, and probably

easier to administer than sodiumbutyrate. In addition, Sigma-Aldrich explicitly stated that tributyrin was food-grade, something not applicable to their sodiumbutyrate or butyric acid products. To our disappointment however, the tributyrin product had a horrifying taste, which, as we later began to understand was probably caused by the remaining 3% of the product. Because the taste was so extreme, we would not dare to administer it in its crude form to any living being, before trying to improve its tolerability, posing us with a major difficulty. Switching to another of our four alternatives would not be a good option either, because these are not really fit for human consumption, as well.

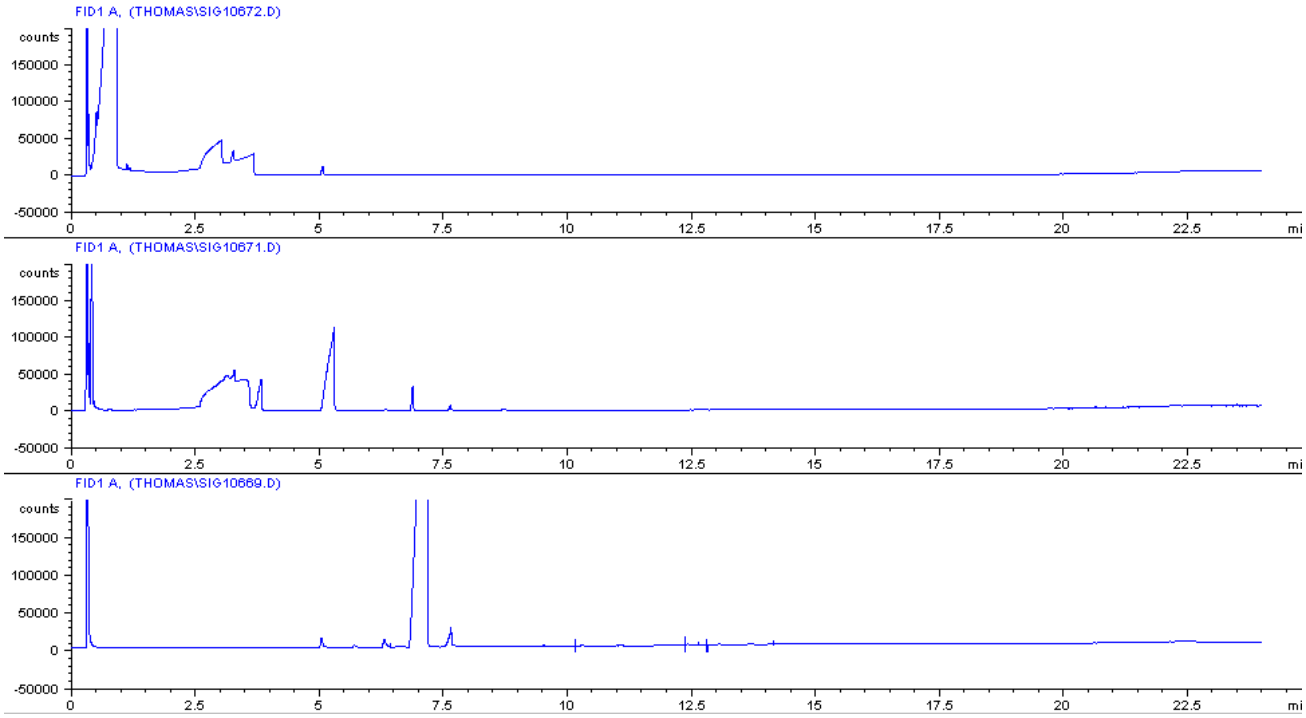
So we decided to contact all companies in the Netherlands we knew capable of encapsulating oils in softgels. All of them required us to take a minimal order of ~50.000 caps – at least ten times the amount we needed, so not an option either. Our next try was to identify the molecules responsible for the taste of the tributyrin, which had over 97% purity. Because triglycerides in general have a neutral to pleasant taste we, as already touched upon, concluded that it was likely that the remaining 3% of it was causing us so much distress – and to find strategies for purification of the oil – of which a report can be found in the later on section.

The next step was to obtain butyric acid and ethylbutyrate from Sigma-Aldrich, just to investigate if it would be possible to chemically or enzymatically synthesize tributyrin from its components: 4 carbon molecules and glycerol. This report is the final one of a series of experiments.

Acid synthesis of mono- di- and triacylglyerols from butyric acid and ethylbutyrate

Background: Previous experiments at Dr. Ten have indicated the formation of glycerolesters of butyrate (monobutyrin, dibutyrin, tributyrin) with the use of enzymes (TLIM, 435 Lipozyme), as indicated from crude use of gaschromatography. From literature we learned that a higher free fatty acid-to-glycerol ratio is needed (>5) to obtain higher yields, as well as the use of higher enzymatic activity (>5% wt/wt). In this experiment however, we used another possibility for the synthesis of triglycerides: perform the procedure under acid conditions (1% addition HCl).

The figure below shows the three GC-spectra for crude tributyrin (Sigma-Aldrich, lower part), butyrate + glycerol + HCL (in the middle) and ethylbutyrate + glycerol + HCL (upper part), which we obtained earlier on. It clearly shows that the tributyrin is contaminated with other molecules, some of which are in the proximity of the tributyrin peak itself.



This experiment sought to perform the previous experiments more thoroughly, with a focus on the chemical sythesis and butyrate, instead of enzymatic synthesis. Alternatively, was used ethylbutyrate as a source, because we expect higher yields with the former as compared to enzymatic synthesis.

Experimental set-up: Butyrate and ethylbutyrate were mixed in glassware in equimolar amounts with 0.1 mole of glycerol (9.2 grams), which equalled 51 and 71 grams of butyrate and ethylbutyrate, respectively. 0,37% HCl (1,70 and 2,40 grams) was added to achieve 1% HCl wt/wt.

After mixing, both were placed in a stirred and pre-heated oil-bath at a temperature of 100 C. This was done to evaporate water and alcohol, which could start forming during esterification and disturb the equilibria of the reactions.

Weight of the two mixtures was:

1) 71 grams ethylbutyrate + 9,2 grams glycerol + 2,4 grams HCl = 82,6 grams of mixture
2) 51 grams of butyric acid + 9,2 grams glycerol + 1,7 grams HCl = 61,9 grams of mixture

Adding the HCl to the ethylbutyrate mixture caused a rapid, but short-lived cloudiness of the reactants. This then was heated for 24 hours.

Results: After approximately 24 hours, the reaction was ended and the mixtures were subjected to neutralization with NaOH, which was, due to my lack of experience, added in abundance in the butyrate flask until a pH=7 was reached, as determined by pH-paper. This not only cleared H+ ions from the HCl, but began to react with the reaction products (supposed butyrates and its esters), thereby forming, as we suspect, sodium butyrate, a soapy, salty tasting substance taking the middle between salt and fat in texture, melting upon warming it between the fingertips.

In the ethylbutyrate-flask (Picture 3), apparently three layers were formed. The upper being coloured slightly blue by the pH-paper and fluid, smelling exactly like ethylbutyrate. The lower layers completely mixed upon gentle handling of the flask, and GC analysis indicated that a considerable amount of tri-,

di- or monoacylglycerols was formed.

Of note: tasting the sodiumbutyrate have prompted me to think that the tributyrin we obtained from Sigma-Aldrich contains a considerable amount of this substance, as both substances share this bitterness and as I am suspecting right now: soapy aftertaste.

Conclusion: Acid synthesis of acylglycerols from ethylbutyrate and butyrate and glycerol is possible, although the reaction with our limited lab equipment does not proceed to the required level.

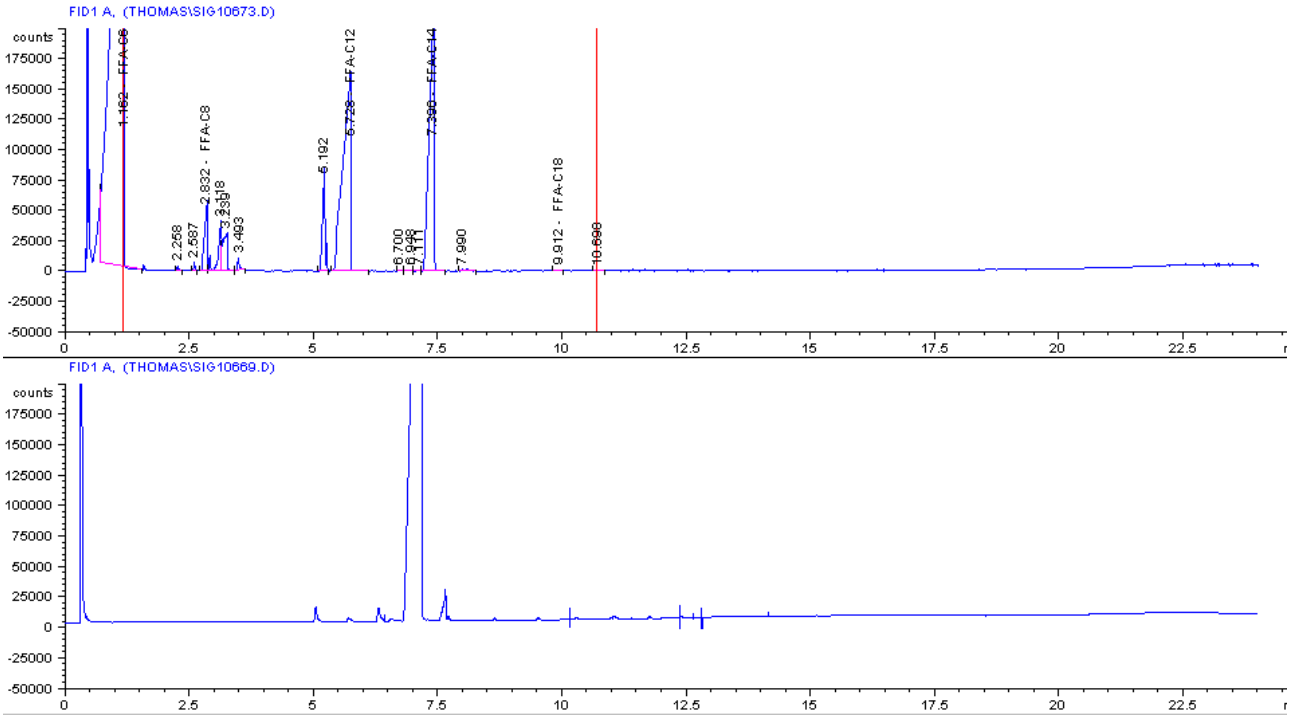
Acid synthesis of butyric acid

Background: Upon examining the earlier GCs, I have decided to zoom-in more on butyrate, instead of ethylbutyrate, for the main reasons that butyrate showed higher TB yield on the GC.

A new reaction was started, in order to shed more light on the benefits of a better reaction procedure.

Methods: 0.2 moles of glycerol (18.4 grams) was were with butyrate in a 1:6 molar ratio (106 grams of butyric acid) and 1% HCl. The mixture was heated at 100 degrees centigrade for the duration of 72 hours, three times as long as than before. After 72 hours, no layers were observed in the mixture, which was homogenous and very clear (glass-like). The apparent viscosity was lower than that of glycerol (see picture). The liquid had the typical vinegar-like smell of butyric acid. PH was ~3.

GC analysis



Gaschromatography revealed the formation of MAGs, DAGs and TAGs from glycerol and butyric acid. The lower graph resembles tributyrin. The yields however, do not seem to be very high.

Conclusion: Again, this reaction confirms that even a longer experimental time and an abundance of butyric acid over glycerol does not result in very high amount of MAG, DAG and TAG formation of the two substances using our limited resources.

Discussion: Encapsulating is not an option, synthesis does not give the expected results, maybe we should try to purify tributyrin?

Purification of Tributyrin 97%

Background: Upon performing GC-analyses of tributyrin and comparing its taste with pure preparations of several fatty acids, we suspected contamination with sodiumbutyrate and several mid-chain length fatty acids. After various failed attempts using different purification methods (crystallization with butter, washing with alcohol, base treatment etc.), we decided to give cooking at 220 degrees centigrade during underpressure a chance. By doing so, we hoped that we could separate the tributyrin (boiling point ~300 degrees Celcius) from the fatty acids (C6:0 and higher), which all have a boiling point <240 degrees. An underpressure lowers the boiling point.

Methods (materials in italics): The *tributyrin* was heated thoroughly in an *oil bath*, whilst at the same time an underpressure of ~850 mbar was generated within the *flask* using a *vacuumpump*. The flask was rotated to allow for the creation of a small film in the upper part of the flask. This ensured rapid evaporation.

Experimental set-up

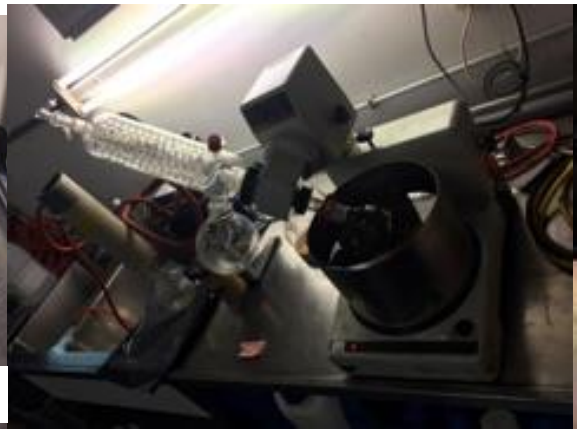
Underpressure

In between the pump and the heated flask, a *column* was placed through which water was shuttled to allow for condensation of the evaporated gases. The condensed liquid then was collected in a *collection flask* connected to the lower part of the column. See pictures below.

Results: After heating the tributyrin in the oil bath for about one hour, and letting it cool down for another hour, I tasted the tributyrin, which still had its pungent soapy taste. The typical free fatty acid taste however, was – at least in my humble opinion – less. This taste, however, was relocated to the collecting flask, in which a layer of clear liquid was formed.

Follow: up: I then undertook a study in how to wash the sodiumbutyrate from the oil. We had previously tried this with plain water, but according to the datasheet of Sigma-Aldrich, only 0,1 g NaB dissolves in 1 L of water. Washing it with PBS however, would theoretically result in a much higher (10 g/L) dissolution. Therefore, I prepared a 500 mL PBS solution by mixing it with 4.0 grams of NaCL, 0.1 grams of KCl, 0.7 g of Na_2HPO_4 and 0,12 g of KH_2PO_4. pH was ~6-7.

Subsequently, I used it to wash the tributyrin by thoroughly mixing it in glassware. After that, I waited until clear-layerforming occurred. This, however, never really happened: the tributyrin droplets were



Experimental set-up

contained in the water, and pipetting it out of the water seemed impossible. A GC analysis was not possible either, because somehow our GC became dysfunctional, and appeared non-fixable.

Conclusion: For this moment, with the equipment and limited experience we have, it appears impossible to purify tributyrin to an acceptable level of taste.

Micro-encapsulating various fats and oils into powders

Goal: To micro-encapsulate various oils and fats into a powder with acceptable taste and potential commercial value.

Background: In October, we visited Bodec, a company involved in biochemical manufacturing and engineering to use some machinery capable of micro-encapsulating various liquids, including emulsified oils. However, the powder obtained at that time contained a substantial amount of free fatty acids, causing it to taste disgusting, probably because of a too long lag time between emulsification and micro-encapsulation, giving destabilized triglycerides to hydrolyse. This time, we opt for the emulsification at Bodec, so that the time between until actual micro-encapsulation is reduced from ~30 hours to ~10 minutes. For economic reasons it was decided not to subject tributyrin to micro-encapsulating, because it would be unlikely the taste would be masked by it, as the previous experiment had indicated free fatty acids or generally not masked by this procedure.

Materials and methods: On the days before encapsulation I prepared all the liquids that were to be encapsulated. In addition, we received a stevia-extract from one of an companion for co-treatment. All in all, ethyl esters of butter oil and fish oil, and inter-esterified butter-coconut blend and a liquid extract of *Stevia rebaudiana* leaves were prepared.

The ethylesters were synthesized by mixing 350 grams of either butter oil or fish oil (obtained from VariaVis) with 200 grams of food-grade ethanol (obtained from Brenntag, Loosdrecht). This 12:1 molar ratio ethanol:oil mixture was heated for 3 hours at 70 °C to allow for the formation of ethyl esters. 1% NaOH wt/wt was added as a catalyst. Shortly after adding the alkali, the mixtures that were a little blurred beforehand turned very clear. This happened during the treatment of both oils. The treatment of the butter oil led to a fruity taste, possibly because of the formation of shorter-chain fatty acids, of which the ethyl esters are used in food processing as flavourings and aromatic substances. After 3 hours, temperature was raised to >100 °C in order to evaporate the remainder of the ethanol. Upon cooling, a jelly-like substance was our end-result, ready for micro-encapsulation.

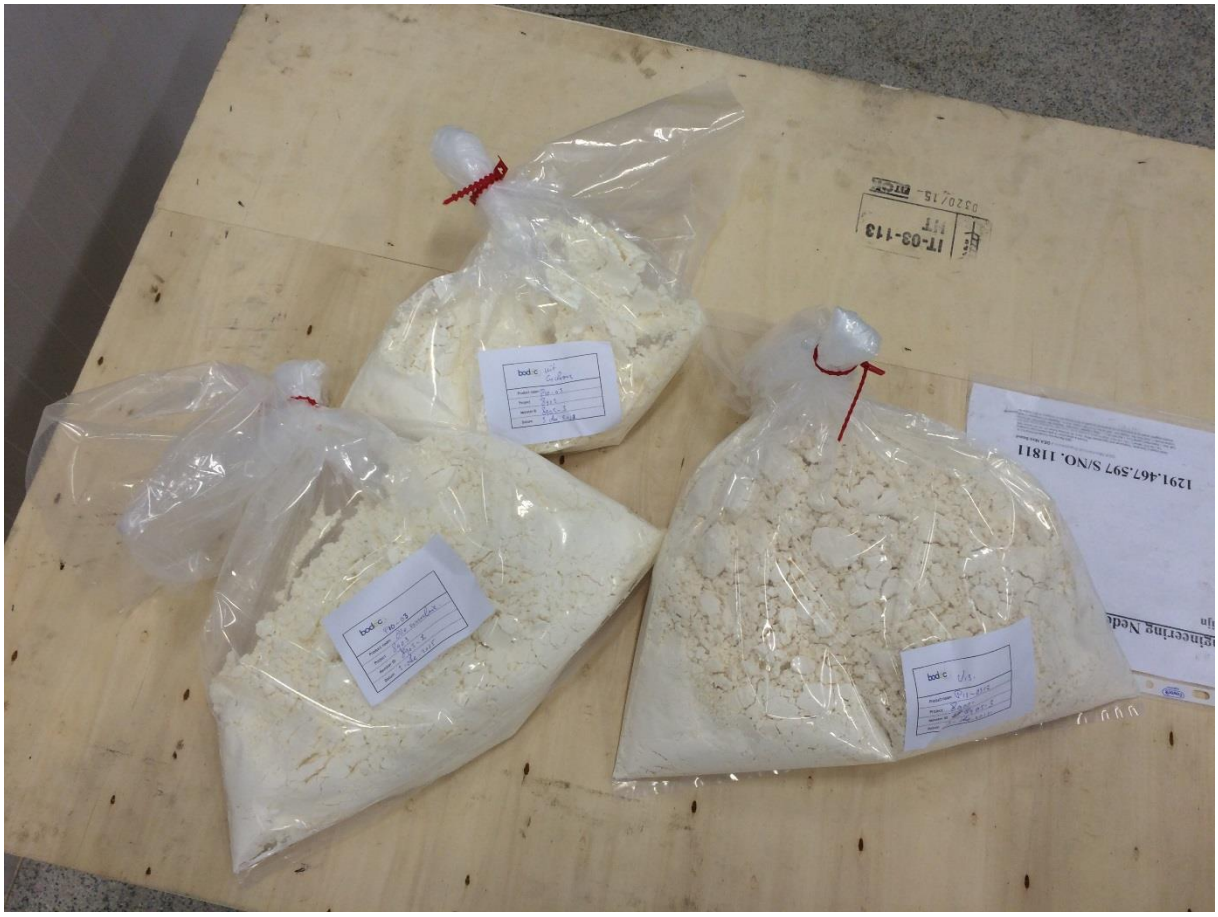
Subsequently, an interesterified blend of butter oil and coconut oil was prepared, by heating both oils for three hours at 60 °C and addition of Lipozyme TLIM, an enzyme suitable for trans-esterifying free

fatty acids. 1% vitamin E as an anti-oxidant was used. The whole reaction was performed under low oxygen tension by conducting a nitrogen flow through the mixture.

Shortly thereafter, they were led through the micro-encapsulation tower, showing up at the end of a long circuit as a fine powder. (Due to secrecy policy at Dr. Ten, I am not allowed to report on the composition of the emulsifiers)

Results and discussion: In contrast to the previous round of micro-encapsulation, this time, the obtained powder had an almost pleasant taste, which can probably be attributed to the short period between emulsification and encapsulation.

Conclusion: It is possible to micro-encapsulate emulsified fish and butter oils, as well as aqueous stevia extract. The amount of free fatty acids that is formed during the procedure is reduced with a shorter time lag between emulsification and micro-encapsulating.



Aanbeveling t.a.v. kleine C-atomen: SCFAs en ketonen (Dutch)

I was asked to do some future recommendations in regards to the use of butyric acid and (possibly) ketones for commercial use.

Verkrijgbaarheid boterzuur en butyraatzouten

Boterzuur is op de markt, commercieel alleen in de vorm van een zout: kalium, natrium, magnesium of calcium (zie links). Het aandeel boterzuur is dan ongeveer 100-500 mg per capsule. Bulkafname van natriumbutyraat is ook mogelijk via Alibaba.com etc.

http://eu.iherb.com/Cardiovascular-Research-Ltd-Butyric-Acid-90-Capsules/59213
http://eu.iherb.com/Nutricology-ButyrAid-100-Tablets/8510
http://eu.iherb.com/Allergy-Research-Group-ButyrEn-100-Tablets/35060
http://www.amazon.com/BodyBio-E-Lyte-Sodium-Butyrate-caps/dp/B0058A9SF0
http://www.amazon.com/Pharmax-Butyrate-Complex-90-vcaps/dp/B0037V3WTA

Nadelen en alternatieven

Het grote probleem met boterzuur en derivaten zijn de geur en de smaak. Een poeder waarin deze stoffen zijn verwerkt zal zeer waarschijnlijk op korte termijn gaan geuren, om van de slechte smaak maar te zwijgen. Dit effect treedt vermoedelijk ook op bij micro-encapsulatie. Camoufleren en bijmengen zal de smaak vrijwel zeker niet kunnen verhullen.

Encapsuleren in softgels lijkt daarom een goed alternatief, hoewel het innemen van grotere doses wel moeilijker wordt, omdat één capsule slechts maximaal 1200 mg boterzuur(derivaat) bevat. Mochten we willen encapsuleren, dan dienen we ons te beperken tot (de vloeibare varianten van) boterzuur.

Een andere optie, is om de tributyrin over een silica kolom te leiden. Dit zou een vrijwel complete purificatie tot gevolg moeten hebben, ten koste van een substantieel verlies aan grondstof. Daarbij komt dat zelfs minieme fracties aan vrije vetzuren leiden tot smaakbederf.

Inkopen of zelf synthethiseren?
Het moet in theorie ook mogelijk zijn om boterzuur te isoleren uit boter. Helaas is het aandeel in boter slechts ~4%. Hydrolyse met zuur of loog, verestering met glycerol of ethanol en/of isolatie d.m.v. centrifugeren of kristallisatie lijken relatief eenvoudige opties om boterzuur te isoleren en om te zetten in zijn zuivere triacylglycerolen of ethylesters. Het grote voordeel van ethylesters is de sterk verbeterde geur: mocht een consument een softgel openen, dan komt hij niet voor de verrassing te staan die zuivere boterzuur of 97% tributyrin kunnen geven.
Bacteriële fermentatie van suikers tot boterzuur is voorlopig iets waar we totaal geen ervaring mee hebben.

Voorlopige conclusie
De vraag rijst of de kosten van deze processen opwegen tegen het inslaan van boterzuur(derivaten) bij Sigma-Aldrich en/of het encapsuleren bij onze encapsuleerder. Persoonlijk zou ik deze laatste optie aanbevelen, omdat deze niet arbeidsintensief is en niet direct de aanschaf van dure apparatuur vergt. Zelf synthetiseren van tributyrin (verestering), butyraatzouten (verzeping), ethylbutyraat (ethanolyse) en boterzuur (fermentatie) is in alle opzichten lastiger en waarschijnlijk duurder. Zie tabel.

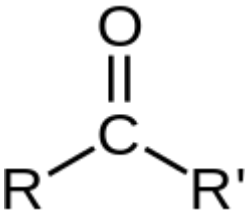
	Aandeel boterzuur	Sigma Aldrich: FG?	Geur	Prijs	Zelf synthetiseren	Toxiciteit
Boterzuur	100%	Ja	Oude kaas/zweetsokken x10	~100 E/10 kg	Fermentatie, geen ervaring mee	Geen
Tributyrin	73%	Ja	Weeige geur	~150 E/5 kg	Mogelijk. Methode vinden voor hoger rendement	Geen
Ethylbutyraat	76%	Ja	Fruitig, alcohol	~180 E/5 kg	Alcohol behandeling van boter -> extractie/kristallisatie. Laag rendement	Geen
Natriumbutyraat	79%	Andere supplier	Lichte geur zoals boterzuur	?	Hydroxides van Mg, Na, K of Ca laten reageren met boterzuur	Niet FG

Alternatief
Desondanks blijf ik de klinische potentie van korte C-verbindingen als mogelijke nutraceuticals onderkennen. Het op de markt brengen van boterzuur in welke vorm dan ook is wat mij betreft nog steeds een welkome aanvulling. Dit hoeft niet alleen te zijn als sportvoeding.

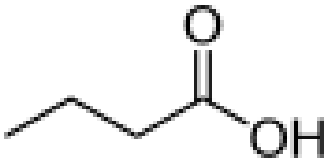
Darmaandoeningen: Boterzuur wordt ingezet bij de behandeling van de ziekte Crohn en Colitis (samen IBD), en heeft een farmacologisch effect (ontstekingsremmend).
*Sport***:** Short-chain fatty acids hebben zeer waarschijnlijk een effect op de vetverbranding. Deze effecten zijn direct, maar ook *indirect*. Dat wil zeggen: niet de verbranding tijdens beta-oxidatie is verantwoordelijk, maar de effecten van SCFAs op genexpressie en eiwitsynthese van cruciale factoren in het metabolisme.
HDAC: Kleine vetmoleculen of vergelijkbare moleculen met een ketogroep verlagen de activiteit van *histone deacetylase*, een complex molecuul dat in grote mate betrokken is bij genexpressie in elke cel van het lichaam. Verschillende medicijnen die op deze werking berusten zijn al geruime tijd op de markt (Voronistat etc.) en kunnen worden ingezet bij een heel scala aan ziektebeelden: Parkinson, Alzheimer, diabetes type 2, obesitas, vrijwel alle vormen van kanker, schizofrenie, depressie, sarcopenie (verlies van spiermassa in ouderen), autisme, epilepsy enzovoorts.

Het gerucht gaat al geruime tijd dat Team Sky van meervoudig Tourwinnaar Chris Froome een sportdrank ontwikkeld heeft op basis van ketonen, structuren zeer nauw verwant aan boterzuur en met vergelijkbare werking. Ketonen hebben minder zuurstof nodig per ATP-molecuul dat wordt geproduceerd dan vetten, koolhydraten en alcohol[24]. Bij een lagere zuurstofopname wordt dus meer energie (vermogen) vrijgemaakt. Als zodanig bieden ook ketonen, met name het pseudo-keton

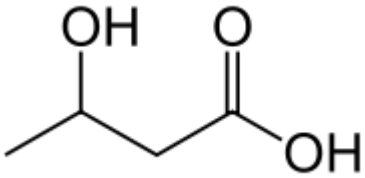
beta-hydroxybutyraat (BHB), een niche in de sportvoedingswereld. Bovendien is ook BHB een HDAC-inhibitor[25].



Ketogroep



Boterzuur



Beta-Hydroxy-Butyraat

Ondanks de grote hoeveelheid mensen die interesse hebben in het gebruik van ketonen of het volgen van een ketogeen dieet is er tot op de dag van vandaag voor zover ik weet maar één commercieel leverancier van food-grade BHB, Prototypenutrition.com: 75 $ per 500 gram BHB-zout. Aceton en acetoacetate zijn overigens te instabiel voor consumptie. De vraag is groot, maar het aanbod laag, zeer waarschijnlijk omdat het zo lastig is om ketonen te produceren. Desondanks is het wel de moeite waar om eens te zien of er toch niet een relatief eenvoudige manier is om daar toe te komen.

Sigma biedt ethyl-beta/3-hydroxybutyraat (CAS 5405-41-4) aan. De prijs ligt bij afname van <5 kg in dezelfde orde van grootte als die van Prototypenutrition.com. Zuur of base transesterificatie zou in theorie moeten leiden tot de volgende reactie, waarin R'OH = glycerol, R"O-(C=O)-C-R = ethylhydroxybutyrate, welke reageren tot R"OH = ethanol + R'O-(C=O)-R = tri-3-hydroxybutyrin:

Deze reactie zal echter zeer waarschijnlijk ook bijproducten opleveren, als de beta-hydroxy of dubbelgebonden O reageert.

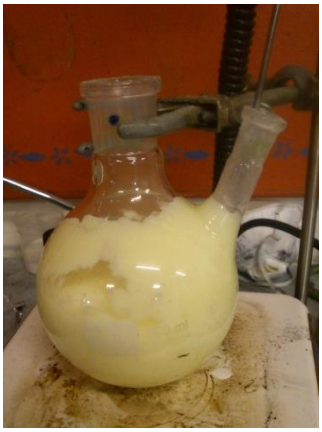
Een andere optie is de vervaardiging van BHB uit poly-hydroxybutyrate (PHB), een bio-degradable plastic dat bij alkali behandeling uiteenvalt in BHB en crotonic acid, een toxisch vetzuur.
Ook zou het in theorie mogelijk moeten zijn om de triglyceride van BHB te synthetiseren. Deze is vindt een mogelijke toepassing in panenterale voeding[26]. Groot voordeel van deze synthese is dat het product wateroplosbaar is en niet de nare smaak van de restproducten in tributyrin met zich mee zal dragen omdat ethylesters in tegenstelling tot vrije vetzuren en zepen doorgaans een fruitige smaak hebben, zodat encapsulatie misschien niet eens nodig blijkt. De grote uitdaging blijft desondanks de manier van synthese en het rendement daarvan.
Ook zou het bacterieel geproduceerde PHB een geheel nieuw prebioticum kunnen vormen. Verschillende studies hebben de non-toxiciteit en positieve effecten op de microbiota al aangetoond, zij het in dierstudies [27, 28].

Toxiciteit
Toediening van SCFAs en ketonen is normaal gesproken veilig. Het zijn lichaamseigen stoffen, waarvan een concentratieverhoging schijnbaar louter positieve aspecten met zich meebrengt. Tot

dusver zie ik in de literatuur nergens serieuze nadelen van deze moleculen binnen de grens van 10 gram per dag.

Verkennende experimenten
Alkaline synthesis of sodium salts of ethyl-3-hydroxybutyrate: 1 mole of non-acqueous NaOH (40 g) was added to 1 mole of ethyl-3-betahydroxybyrate (123 g) as a preliminary investigation into future experiments. The mixture was heated at 100 °C in order to evaporate the possible ethanol that would form when free-flowing ethylgroups would react with the OH^- molecules. We hoped that alkaline hydrolysis using NaOH would result in the positively charged sodium-ions reacting at the site

were the ethyl groups were to be chopped off, thus forming sodium-3-hydroxybutyrate. Soon after the initiation of the reaction in glassware, a milky substance formed, consequently turning into a foam, out of which, seemingly, a lot of evaporation occurred, ultimately leaving thick salty-soapy grains, as shown on the right.




Study participants

Despite our problems with the to be administered compound, we contacted iSkate, a professional skating team in Heerenveen and potential supplier of study participants (iSkate.nl). They were eager to volunteer in a potential clinical stuy, and to our great satisfaction the participation of a total number of 18 potential volunteers, well-trained speed skaters ('Jong Oranje'), was assured. Although our initial idea was to ask Isala for help and participants, we now were allowed to operate in a much more professional environment. A detailed study proposal was presented to iSkate, which is schematically reproduced here in Dutch.

Unfortunately, over the course of October, it became apparent that, due to their busy training schemes and the start of a new competitive speed skating season ahead, iSkate had decided to withdraw from participation. This came as a big surprise, and so after two months of work we still empty-handed: no suitable product to test, no participants. Therefore, Plan B was initiated: collaboration with Isala. Some of their sports physicians were willing to give assistance during the eventual testing, as well as making available experimental apparatus. We then sent various emails to cycling teams in the vicinity of Zwolle, in order to obtain volunteers. Isala contacted participants of trials that were performed earlier on. Again, we encountered a disappointment: virtually no response to all the efforts we made in the recruitment. This forced us to turn to our own staff and relatives as participants, maybe they were willing to participate? At least a facility was available, and, to our surprise, we were offered the option of encapsulating a small batch (5000 softgels) at an encapsulation facility in Belgium. Some of our customers had personally approached the CEO of the company, to which he was familiar, thereby opening a critical door to the execution of the study. By then, it already was late November, and upon contacting the encapsulation company, it became apparent that 2015 would not become the year with an intervention, although we now had an excellent picture of all possibilities, drawbacks and limitations, so that a future study can be started-up very soon.

Rationale: Tributyrin in exercise performance

In the beginning of 2016 it became clear that it would be feasible to start a small pilot study with 10 of our employees and volunteers at Isala. Beforehand, I was asked to review my initial rationale and to adapt it according to the wishes of Dr. Ten and the Dutch government, an excerpt of which can be found here.

Ergogenic potential of medium-chain triglycerides

In the past, various short and long-term studies have tried to gain insight into the ergogenic potential of medium chain-length fatty acids[1, 10-16], which need not to be incorporated into intestinal chylomicrons for absorption, but instead rely on diffusion across the intestinal epithelium, upon which they are transported directly to the liver and can be released into the bloodstream as free fatty acids. The purported ergogenic potential of these so-called mid-chain triglycerides (MCTs) as compared to their longer-chain analogues mainly comprises their ability to increase the absolute and relative rates of fat oxidation, because they can directly cross the cellular and mitochondrial membranes and hence, do not saturate carnitine-palmitoyl tranferase (CPT-1), the rate-limiting enzyme for fatty acid entry into mitochondrial beta-oxidation[1]. By doing so, they would add and contribute to total non-glycolytic energy production and dampen glycogenolysis, and promote

increases in aerobic work capacity as indicated by lowered plasma lactate levels, increased times to exhaustion and decreases in respiratory exchange ratios during exertion[1, 10-16].

Disappointingly however, scientific conclusions on the potential of the MCTs are highly ambiguous, with short-term/acute administration and/or simultaneous carbohydrate feeding during the tests appearing ameliorative to their effects, whereas a trend towards benefit is observed with long-term administration[1, 10-16]. Only one placebo-controlled human intervention yielded increased times to exhaustion in recreationally active athletes after the administration of 6 grams of MCT-oil for two weeks[1], whereas one human short-term study concluded that MCTs increase endurance capacity, although only in the absence of carbohydrates[14]. It is suggested that the effects of longer-term administration may be brought about by the stimulation of enzyme activities of the TCA cycle and/or ketone body utilization, some sort of 'hormetic' effect that needs at least a few days before showing up[15].

Accordingly, the primary aim of endurance training is to force – and learn - the body to spare glycogen (carbohydrate) at the expense of using the virtually unlimited storage of triglycerides (fat) in the adipose tissue[17]. As such, the effects of endurance training rely on the body *super-compensating* to the artificially created state of energy depletion during training, mimicking starvation, or a low-carbohydrate e.g. ketogenic diet by improving aerobic energy generation[17]. The increased oxidative capacity in endurance athletes not only facilitates a higher power output for given heart rates, it allows the body to rely more on fatty acid oxidation at higher exercise intensities, that is: athletes' *respiratory exchange ratios* are lower at the relative utilization of their *maximal oxygen consumption* when compared to untrained individuals. The RER is strongly linked to exercise performance, as VO2max and lactate threshold determine more than half its variance[18]. Therefore, for maximal performance, the body's ability to oxidize high absolute amounts of fatty acids seems key. The so-called *train-low-compete-high*-adagium finds its roots in this principle, and means that an athlete should aim for maximal lipolytic-oxidative capacity by training in a glycogen depleted state, so that the body adapts to the increased demands on its oxidative metabolism by reliance on it even during more glycolytic and/or gluconeogenic activity[17].

What about the short-chain fatty acids?
If boosting the body's ability for beta-oxidation is one of the major adaptations to training and a prerequisite for top-level performance, and supplemental consumption of MCTs as compared to longer-chain fatty acids partly stimulates this process, why not try to do so with the use of the even shorter-chain fatty acids: acetate, propionate and butyrate, one may ask.

Accordingly, it is found that '*Acetic acid enhances endurance capacity of exercise-trained mice by increasing skeletal muscle oxidative properties*[8]. *Acetic acid significantly increased the muscle expression of key enzymes involved in fatty acid oxidation and glycolytic-to-oxidative fiber-type transformation. Taken together, these findings suggest that acetic acid improves endurance exercise capacity by promoting muscle oxidative properties, in part through the AMPK-mediated fatty acid oxidation and provide an important basis for the application of acetic acid as a major component of novel ergogenic aids*[8].*'*
This publication is the only one so far that deals directly with the use of a short-chain fatty acids in endurance capacity. Additionally, it is no human intervention, although it gives a clear indication for the mechanism of action of acetic acid: it somehow activates, be it directly or indirectly, the 5' adenosine monophosphate-activated protein kinase (AMPK), a sensor for cellular energy status, and

one of the major mediator for the adaptations commonly observed in response to endurance training[19].

Metabolic mediators of training stimulus and adaptation

When cellular ATP levels plummet and AMP levels rise, as happens during exercise, AMPK is activated[19]. This not only directs the glucose transporter 4 (GLUT4) to the cell membrane for insulin-stimulated glucose clearance from the circulation for glycogenesis, it also promotes ketogenesis and skeletal muscle fatty acid oxidation, so that glycogen stores are preserved and total aerobic capacity is increased[19]. To allow for higher oxidative capacity, the organism not only needs to increase its oxygen uptake and delivery capacities, the cellular capacity *per se* for higher oxidative rates needs to be ramped up too. To meet these demands, mitochondrial density and quality are to be increased, because oxidative (non-glycolytic) metabolism takes place in the mitochondria. Accordingly, another target of AMPK is the *peroxisome proliferator-activated receptor gamma coactivator-1-alpha* (PGC-1α), an integrator of cellular energy status and master regulator of mitochondrial biogenesis [20]. Thus, by artificially raising the AMP:ATP status in the cell through exercise, AMPK (and PPAR-γ) orchestrate the cascade that leads, via PGC-1α, to increased mitochondrial oxidative capacity: *'*In vivo *ectopic expression of PGC-1α in skeletal muscle not only induces mitochondrial biogenesis and OXPHOS activity, but also switches type IIb and IIx/d glycolytic fibers to type I and IIa oxidative fibers. As a result, the transgenic mice have improved endurance running performance*[5]*.'*

As opposed to artificial energy-depletion, pharmacological activation of AMPK (e.g. by AICAR, R419) and PPAR-gamma (e.g. by GWs, thiazolidinediones) can enhance endurance capacity:

'...AMPK-PPAR-δ pathway can be targeted by orally active drugs to enhance training adaptation or even to increase endurance without exercise[3].'
'…the group submitted to both exercise mimetics [AMPK and PPAR agonists] and exercise training presented improved functional performance (…) as well as aerobic capacity[4].'
'...PPARβ/δ agonist and exercise training synergistically increase oxidative myofibers and running endurance in adult mice[3].'

These substances now are prohibited by the World Anti-Doping Agency, and several athletes have been suspended for its use[21-23].

The case for histone deacetylase inhibitor butyrate

No literature was found for the effects of propionate and butyrate on endurance capacity. However, supplementary sodiumbutyrate at 5% wt/wt of a high-fat diet (HFD) given to rats has been shown to increase fatty acid oxidation (illustrated by the decreased RER and increased energy expenditure) and adaptive thermogenesis, whilst gains in body fat are completely diminished[2]. Most strikingly, mitochondrial function and biogenesis in brown fat and skeletal tissue increase as well, as are the levels of type I ('slow twitch') muscle fibers and levels of myoglobin. This finding probably finds its roots in the upregulation PPAR-γ coactivator-1α (PGC-1α), the levels of which are increased at both the mRNA and protein level[2]. Levels of AMPK are also upregulated by butyrate and may explain the enhancement of PGC-1α activity. Additionally, CPT-1b, PPAR-δ and cytochrome oxidase I are increased, whereas plasma triglyceride and cholesterol levels show a decline[2], indicating that butyrate may, just like the MCTs and acetic acid, enhance the bodies total 'lipoxidative' capacity. The authors of the publication propose that all of the effects of butyric acid are to be attributed to its inhibition of histone deacetylase (HDAC), an apparent key (up)regulator of the aforementioned genes[2], which is consistent with the notion that *'Potthoff and colleagues have illustrated that over-*

expression of HDAC5 is negatively *correlated with endurance training-mediated adaptations in mouse skeletal muscle*[6]*',* and that *'...the selective degradation of class II HDACs in slow skeletal muscle provides a mechanism for enhancing physical performance and resistance to fatigue by augmenting the transcriptional activity of MEF2*[7].*'*

Be it their HDAC-inhibiting properties, their regulation of intracellular acetyl-Coa or AMPK levels, a possible upregulation of TCA enzymes, or even other mechanisms, the SCFAs show some interesting characteristics, which seem to justify the notion that they possibly may be able to favourably alter parameters normally associated with enhanced endurance capacity.

Proof of concept on butyrate: a pilot study
Objective: To verify whether the case for butyrate, in addition to acetic acid, is a reasonable one, we propose that a non-blinded proof-of-concept pilot-study is initiated, in which butyric acid is to be administered to healthy, active subjects.
Hypothesis: We here hypothesize that the administration of butyric acid will bring about beneficial changes in fatty acid oxidation (as indicated by RER) and work capacity during an incremental exhaustive VO2max ergometer test.
Intervention: 20 grams worth of tributyrin will be administered 2 hours before an incremental, exhaustive cycle ergometer protocl. Isocaloric amounts of fish oil will be administered in the same dosing regimen in softgels to a placebo-treated group, to compare the effects of butyric acid to the well-established in-vivo AMPK agonistic properties of fish oil[29-31].
Outcomes: Study participants will be subjected to an incremental, exhaustive VO2max ergometer test at Isala Sportgeneeskunde, Zwolle, The Netherlands. Participants will warm-up at 100 W for ten minutes, upon which power increases 40 W every three minutes. Every full minute spent in the last and exhaustive step will be accounted for by 1/3 of the power that was sustained during this step. In conjunction with the measurements of power output, analysis of total and relative breath gas exchange will be used to determine (changes in) VO2max and RER. During the test, lactate will be measured at the transition to an incremental step (t=10, 13, 16 etc.) and at the end of the intervention as an indication of anaerobic metabolism. They will be instructed to not consume any foods or calorie-bearing drinks during two hours before the test and to abstain from caffeine ingestion, as this may interfere with RER values.

Preliminary remarks/discussion
- Our rationale partly relies on *in vitro* and non-human interventions.
- Chronic or sub-chronic administration could be more efficacious as compared to an acute intervention comprising a single large bolus of butyric acid.
- Intervention is non-blinded.
- Our study population is of small size and rather heterogeneous, which may lead to false positive or false negative outcomes due to large variation.
- Possible favourable outcomes will need to be verified in well-trained elite athletes before firm conclusions can be made regarding the ergogenic potential of butyric acid.
- Butyric acid is a preferable source of energy for the enterocyte, although plasma levels of butyric acid are well-documented to be increased upon oral consumption of tributyrin. Apparently, after hydrolysis by brush border lipases, tributyrin-derived butyric is not metabolized by the enterocyte solely, but reaches the liver and increases plasma levels of butyric acid[32, 33].
- Quite recently, lactate has been disputed as a fatigue-promoting substance, but can still be used as a measure of anaerobic (glycolytic) metabolism, which is of interest for our intervention.

Invitation to the subjects (translated from Dutch)

Background

Dr. Ten is developing sports oils and also investigates their effects. Very promising results have been gathered in the past with our sports oils. At the moment, Dr. Ten would like to do some more research into both well-established products as well as into novel fats.

It is known already that certain fatty acids contribute to enhanced endurance. This is because of the relative easiness in which they are used as a fuel source in the body.

Dr. Ten aims to investigate the potential of these fatty acid in endurance capacity by means of measuring various parameters after consumption of the oil (or a placebo).

Aim

It is expected that the oil will enhance the endurance capacity of endurance athletes. This will be measured during an cycling test on an ergometer, which measures power output and work. In addition, analysis of inhaled and exhaled gases will be performed.

Who can participate?

Mature men and women can participate in the study, except for when they have diabetes and use medicine for it. Study participants must come on two occasions to Zwolle to perform cycle ergometer tests. Meanwhile, they consume the softgels containing the oil.

What is expected of the participants?

The project will start Februari 22nd and will last exactly two weeks. During this period, participants consume two different oil products. Every participant will be randomised to either one of the oils and will be uninformed about which oil he is taking.

To test the effects of the product, two cycle ergometer tests will be performed: one baseline measurement and after the intake of the oils.

The testing days

The test will be performed on Monday Februari 22nd and 14 days later, on March 7th, or, alternatively Mondy Februari 29nd and 14 days later, on March 14th. Isala Zwolle is located at the Landstede Sports centre, Hogeland 10, 8024 AZ. A skilled, professional sports physician will guide the participants during the test.

Within two hours before the test, it is forbidden to consume any food or calorie-bearing drinks. It is also prohibited to use any caffein before the tests, so, no coffee before testing! These measures are taken to avoid interaction with the administration.

During the test, every participants cycles for 10 minutes on a workload of 100 W. After that, workload will increase by 40 W every three minutes, just until exhaustion.

Is the product safe?

Dr. Ten makes use of 'food-grade' products exclusively, which means that all products are suited for use in food. The oil that is to be investigated is food-grade too and produced under food-grade conditions under responsibility and approval by Dr. Ten. However, because it is only for the first time that the oil is investigated in this way, it is impossible to predict how big its effects will be. Based on literature, we do not expect any side-effects or harmful health effects, although sometimes gastro-intestinal complaints occur at very high intakes of fatty acids.

In the next coming weeks we will contact you by telephone or email, to see if you are interested. If you have any questions, do not hesitate to conctact Dr. Ten 038-2000153.

Report: Tributyrin in exercise performance

Participants

11 male participants from the vicinity of Zwolle agreed on participation to the study and allocated to either the fish oil or tributyrin treatment. Mean weight of the subjects included in the analysis was 79,2±7,7 kg, age 34,5±6,8. One participants dropped-out due to illness and was excluded from analysis, so that 10 were eligible for analysis: 4 performed their second test after tributyrin ingestion (TB), 6 after consumption of fish oil (FO).

Materials & Methods

All test at Isala were performed on the same ergometer, with the same heart rate and breath exchange analysis device and using the same software analysis.
Fish oil was administered in 17*1200 mg capsules, whereas participants allocated to tributyrin ingested it all at once from a test tube 2 hours before the start of the test. If possible, they performed their tests on the exact same time. Due to occupational or private obligations, this was not possible for every subject.
The protocol consisted of a 10 minute 100 warm-up at 100 W and was followed by an increment of 40 W every 3 minutes until exhaustion or when RPM >70 could not be sustained. At the end of every 3-minute stage and at exhaustion, lactate was measured from a drop of ear-lobe derived blood. Heart rate was recorded continuously, as was power output.

Results

10 out of 11 participants completed both trials, whereas one from the fish oil group indicated that he could not perform well due to a hard week of labour. He was excluded from analysis. One participant from the TB group indicated that he got ill after completion of the test. All participants declared that they had given their all and had cycled until exhaustion. The main findings are presented in the table.

Table 1 Results from the intervention. Significant are p-values <0.05. TB=Tributyrin treated group, FO=Fish oil treated group. P-values are for the comparison of the previous column and the column indicated under 'p-value'. HF=Heart frequency (beats per minute), power is in Watts, VO2 in Liters/minute, RER = VO2/VCO2 (not shown), lactate in mmol/L blood, VT = ventilatory treshold e.g. 3 minute power load in which RER approaches or exceeds 100.

	1. Baseline TB	2. Baseline FO	P-value	3. TB	P-value	4. FO	P-value	P-value
	(n=4)	(n=5)	1 vs. 2	(n=4)	1 vs. 3	(n=5)	2 vs. 4	3 vs. 4
Weight	81.0±2.9	79.3±7.6						
Max_HF	185±9.6	181±11.4	0.594	184±6.0	0.865	180±10.2	0.887	0.513

Max_Power	404±1.8	280±42.7	**0.001**	395±13.8	0.243	279±31.2	0.967	**0.001**
Max_VO2	4.75±0.30	3,48±0.37	**0.001**	4.77±0.16	0.910	3.23±0.38	0.323	**0.001**
Max_Lactate	10.3±0.35	11.7±1.21	0.063	10.3±1,3	1.000	11.4±1.96	0.300	0.369
Max_RER	1.10±0.043	1,14±0,063	0.317	1.07±0.015	0.236	1.14±0.044	1.000	**0.020**
VT_Power	340±20	236±35	**0.001**					
VT_RER	0.99±0.009	1,01±0,030	0.244	0.98±0,019	0.378	1.01±0,026	1.000	0.096
VT_HF	174±7.5	166±9.0	0.198	174±6.0	1.000	164±10.6	0.756	0.139
VT_VO2	4.29±0.24	2.99±0.42	**0.001**	4.35±0.16	0.692	2.93±0.32	0.806	**0.000**
VT_Lactate	6.6±0.88	7.6±1.2	0.208	5.1±0.80	**0.045**	5.5±1.0	**0.017**	0.537

At baseline, both groups did not differ in weight. However, their VO2max and VO2 at ventilatory thresholds (power load in which RER approaches and/or exceeds 1.00) as well as their maximal power outputs and power output at VT were significantly ($p < 0.05$) higher at baseline were higher for the TB group. This effect was still present after oil consuption. (Of note, the power load in which an RER value of 1.00 was approached or exceeded was used in the second cycle test to determine if average RER during this step would be lower, indicating an improvement in oxidative metabolism.)

Fish oil and tributyrin failed to improve all parameters (intra-group) except for lactate concentration at ventilatory treshold (both groups). Additionally, RER values were different between both groups after oil ingestion: maximal RER values during TB ingestion were significantly lower than in the fish oil group, an effect that was not present at baseline.

Conclusions

Acute tributyrin and fish oil consumption at 20 grams 2 hours before an incremental exercise protocol do not appear to affect absolute rates of endurance capacity. However, lactate concentrations the tests appear to be lower after ingestion of these oils. Additionally, maximal RER values were significantly lower after TB than FO consumption, although both values did not differ in respect to their initial control values.

Discussion

Few remarks should be made on both the results of the protocol as the study design itself:

1. The drop-out (illness, TB group) and exclusion from analysis due to pre-existing fatigue (FO) further cecreased the already low power of the intervention, due to the low amount of subjects in each treatment arm.
2. Some of the subjects were not familiar with the whole procedure, a learning effect during the intervention may have occurred, although the data do not confirm this notion directly.
3. The lowering of the lactate levels in both treatment groups as compared to baseline is remarkable. It may be that the pro-oxidative capacities of both fish oil and tributyrin via activation of PPARs (or others) have improved total lipo-oxidative capacity of the body, thereby lowering the needs for glycolytic contribution to energy demands. However, VO2 was not modified in both groups, as were RER values (indicative of anaerobic, glycolytic metabolism), although a non-significant trend was observed in the TB group as compared to the FO group: RER values for given power outputs fell from 0.99±0.009 to 0.98±0.019 ($p=0.096$), whereas the fish oil group remained constant: 1.01±0,030 to 1.01±0,026 ($p=1.000$).

4. It may be hypothesized that a protocol consisting of fixed workload at 90-100% of RER instead of incremental power outputs may be better suited to reveal any differences tributyrin and placebo fish oil. In doing so, substrate utilisation is better studied and the effects of glycogen depletion can be determined. A relatively short incremental protocol is better suited for determining total oxidative and anaerobic exercise capacity.
5. Future studies are needed to draw conclusions on the ergogenic potential of tributyrin as compared to positive and (fish oil) negative control (baseline)

General conclusion and recommendations

The internship at Dr. Ten has given me an insight in the functioning of a possible future employer, and also, learned me how to deal with the procedures preceding a human scientific trial. Particularly the latter was one of my subgoals beforehand and the above report poses a detailed overview of the process. The question whether butyric acid can enhance endurance capacity and/or body composition however, I have not been able to answer because of the difficulties encountered along the road. We have however, a clear view of the (im)possibilities. First, it is not advised to try to synthesize a product ourselves, despite the possible commercial value it would bring about. If the major chemical companies have not been able to make a pure product, why think we could with our limited laboratory equipment? Second: administration of butyric acid is likely most convenient when it is encapsulated in softgels, although this is difficult. The use of machinery is most easy at Isala. The additional help of an certified sports physician is of considerable interest, despite the relatively high costs. Thus, upon encapsulation (1300-2600 euro), finding participants (50-100 euro compensation per person) and performing the tests (~150 euro each), we hopefully will be able to make conclusions on the potential of butyric acid in sports medicine and exercise physiology.

Besides the focus on butyric acid and sports performance, other applications using derivatives of butyric acid are of future interest. Especially the development of new prebiotic fibres derived from BHB is interesting. Preliminary studies already have shown its potential[27, 28]. Several companies sell these compounds, although they generally are indicated for use in cosmetics and come in a rather high chain-length, causing them to be poorly bio-degradable because of relatively low water-solubility.

All in all, after performing our pilot study, no firm conclusions can be drawn on the ergogenic potential of butyric acid. I am sure that an the execution of a methodologically simple and elegant study should be possible, but we have been held back by some practical issues, lack of experience, financial matters etc.

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8. Glycerine valorisatie – glycerol fuel cell

Electrochemical Oxidation of Glycerol and Reduction of Oxygen in Alkaline Media

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Abstract

The electrochemical oxidation of glycerol was studied by voltammetry on different catalyst materials (Au, Pt, Cu, Ag and Glassy Carbon) in alkaline, aqueous solutions. It was found that Au, Pt and Cu have a higher catalytic activity towards glycerol oxidation at 1 M glycerol in 1 M NaOH than other metals, as witnessed by different current densities. The highest current density for glycerol oxidation (10mA/cm^2) was found at -0.2V for the Au electrode. Besides, a change of substrate material for gold deposition has an effect on the current density although it deterioration was not studied yet. From the substrate materials tested (Zn, Fe, Cu, Ni and carbon graphite) it was found that copper coated with gold particles has the highest current density (of 60mA/cm^2) at a voltage of -0.2V. The oxygen reduction reaction in 1M Glycerol and 1M NaOH was studied too and it was found by voltammetry that carbon graphite of different surface areas has a substantial influence on voltage favoring the ORR towards positive voltages. Carbon 2765 (Asbury) was found to have the highest current density when compared with other carbon graphite at a voltage of -0.6V (40mA/cm^2) although no nanotubes and nanoparticles were studied. An attempt to build a new simple fuel cell system for glycerol oxidation was done for the glycerol oxidation and the ORR using Cu+Au as anode and carbon 2765 as cathode materials. The maximum current density discharge that the prototype can have is -0.35mA/cm^2 in 1M glycerol + 1M NaOH.

Key words: Voltammetry; graphite; glycerol

1. Introduction

Fuel cells are an appealing technology that can be used as back-up power in off-grid scenarios [1]. Solar PV and wind energy can produce electricity in sunny and windy days accordingly, and the surplus of energy can be stored in batteries [2]. However, during cloudy days in winter or low wind days the energy supply can be compromised [3]. An alternative to give back up power could come from fuel cells.

Fuel cells can convert directly a fuel into electricity [4]. One common fuel use in fuel cells is H_2 and its production is well known in chemical production plants. However, to store hydrogen is still a challenge due its high volatility and safety concerns [5], one alternative to the problem is to use a fuel that is liquid in ambient conditions. There are different types of fuels that have been tested in the past as possible alternative fuel such as, hydrazine, organic compounds and formic acid [6] [7] [8].Nevertheless, there are technical and commercial necessities that a fuel most comply in ordery to be use in a fuel cell e.g. availability, transport, safety and cost. Taking this into account, the quantity of fuel possibilities is reduce to a few types [9]. One organic material that is of interest is glycerol. Glycerol ($C_3H_8O_3$) is a sub-product of biodiesel production, it is expected that in 2018 the production of glycerol will increase in approximately 20%. [10]. Glycerol is use as: food supplement for animals, in the cosmetics industry, and others in less quantity (polyurethane production, pharmaceutics and alkyl resins). However, the current worldwide production of biodiesel is increasing rapidly and generates a surplus of glycerol that needs new alternatives to be use [11]. One alternative to use glycerol is a fuel in fuel cells.

Electrochemical studies have been done on glycerol oxidation in the pass, special attention has been done towards catalyst selection in alkaline and acid media and the following materials have been report: Pt [12], Pt-Ru [13] , Au, Pd [14], C-Ag [15], and MnO_2 [16]. Also studies for ideal membrane for the glycerol fuel cell system has been investigated [17]. While glycerol is oxidize at the anode oxygen reduction reaction (ORR) occurs at the cathode electrode. The ORR has been studied in the pass and review papers have been done describing the latest advances [18] [19] [20], but less studies has been done directed to the ORR in a glycerol fuel cell [21].

This research is pretended to give a better understanding of the electrochemical mechanisms of glycerol oxidation in alkaline media and working at room temperature. The study shows first an inside into possible catalyst for glycerol oxidation and an analysis of different substrates for Au catalyst coating, it also presents the results obtain with commercial carbon graphite materials and its effect in the ORR. Finally, it is show the results obtain in a small prototype showing the discharge effect of a glycerol fuel cell. The analysis has been done by means for cyclic voltammetry and chronoamperometry.

3. Results and discussion

This section shows the results obtained for the glycerol oxidation and the ORR experiments performed during this research, firstly, the CV screening of different catalyst materials for glycerol oxidation is presented (Au, Ag, Pt and Cu) , secondly, the CV analysis of glycerol oxidation using Au coated on different metal substrates (Fe, Glassy Carbon, Carbon Graphite, Ni, Zn and Cu) thirdly, the CV analysis and comparison of the ORR using different commercial low cost carbon graphite, and fourthly, the discharge experiments of a prototype fuel cell at room temperature.

3.1 Cyclic voltammetry screening of catalyst for Glycerol Oxidation

Cyclic voltammetry is used to get an insight in the oxidation of glycerol in alkaline media using different catalyst as is shown in Fig. 3.1. The experiments are run at 100mV/s and between -1V to 1V using Ag-Ag/Cl as reference electrode in 1M $C_3H_8O_3$ + 1M NaOH.

The CV for the catalytic effect of Pt electrode for $C_3H_8O_3$ oxidation is shown in Fig. 3.1a. It can be seen that the current density increases during the positive voltage screening until the max voltage/current is reached, then the forward voltage is applied until a point in which the Pt reduction occurs, the first peak (around -0.3V) corresponds to Pt reduction and the second peak corresponds to $C_3H_8O_3$ oxidation (around 0.1V), these results are in accordance with other authors [13]. Fig 3.1 b and d show the CV of Ag and GC electrodes for glycerol oxidation respectively. The cyclic voltammetry shows the behaviour of Ag and GC electrodes in alkaline media which is similar to other result in literature [22], however, no clear evidence of $C_3H_8O_3$ oxidation is observed. Fig. 3.1c shows the CV of Cu electrode for glycerol oxidation in alkaline media. Three current density peaks at different negative voltages are present (-0.3V,-0.1V and 0.1V). The first two current density peaks (from left to right) might correspond to copper oxidation in different states as shown in literature (Cu^{3+} <> Cu and Cu^{2+} <> Cu) [23],and the third peak can be interpreted as glycerol oxidation.

The CV of Au in 1M glycerol 1M NaOH is shown in Fig 3.1e. It can be seen two current density peaks at -0.2V and -0.1V voltage. The first peak (from left to right) corresponds as typical Au reduction in alkaline media. The second peak could correspond to the $C_3H_8O_3$ oxidation identify by previous authors [14].

In general, the catalytic activity towards glycerol oxidation is observed in the Pt, Cu, and Au electrodes, however, in the Ag and GC electrodes not clear evidence of $C_3H_8O_3$ oxidation is observed. When comparing current densities for $C_3H_8O_3$ oxidation at -0.2V the Au electrode has the highest value 11mA/cm^2 followed by Cu 8mA/cm^2 and the lowest current density observed is from the Pt electrode 4mA/cm^2. This study focuses in the catalytic activity of Au over different substrates. Pt and Cu electrodes are beyond the results fount in this paper.

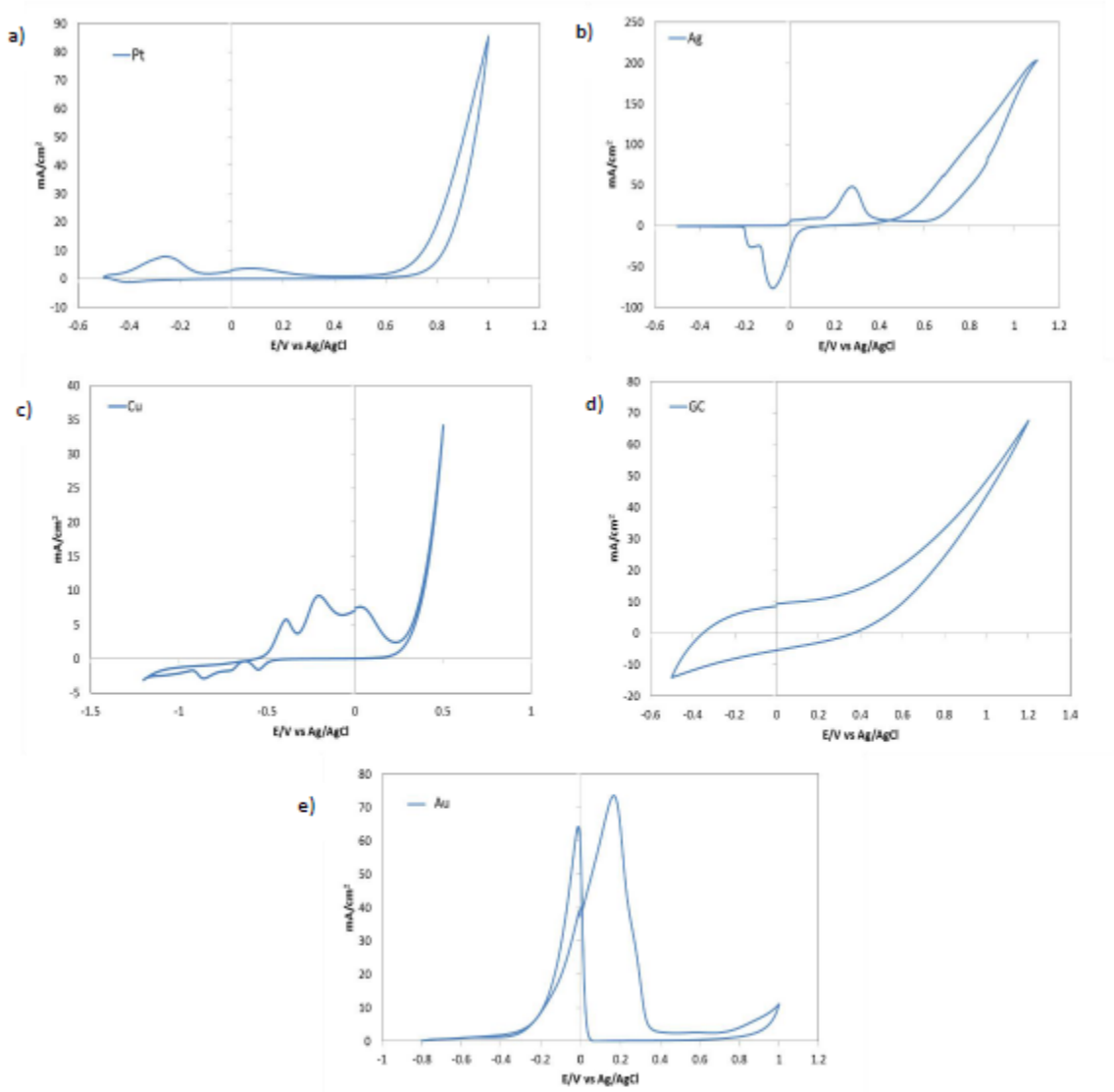


Figure 3.1 Cyclic voltammetry of a) Pt, b) Ag, c) Cu, d) GC and e) Au electrode on 1M $C_3H_8O_3$+1M NaOH electrolyte against Ag-AgCl reference electrode, 100mV/s and Ag-Ag/Cl reference electrode.

3.2 Effect of pH on glycerol oxidation using Au catalyst.

The effect of pH in the oxidation of 1M $C_3H_8O_3$ is studied using cyclic voltammetry. The electrode use is coated Au on glassy carbon (GC) electrode in acid (1M $C_3H_8O_3$+1M H_2SO_4) and alkaline (1M $C_3H_8O_3$+1M NaOH) media. The study is performed at 100mV/s and with Ag-AgCl as reference electrode. The coating in the GC electrode is performed as explain in section 2. Fig. 3.2 shows the electrode of 2mm diameter with Au coating (Fig.3.2a) and without Au coating (Fig.3.2b).

The CV of GC + Au electrode compared with pure Au electrode in acid media is shown in Fig 3.3a. When the voltage is increase in the positive direction of the scan a first current density peak is observed, possibly, corresponding to Au oxidation, then the voltage is change and Au reduction current density peak is present. This behaviour is typical for Au electrode in acid media [24]. In both cases, GC+ Au and pure Au electrodes, cathodic and anodic current density peaks are present, this is an indication that Au coating is active in the GC material showing similar behaviour than the pure Au electrode, however, it is not observed a clear evidence of $C_3H_8O_3$ oxidation during the experiment.

Fig. 3.3b shows the CV of Au and GC + Au electrodes in alkaline media. The behaviour is similar to fig. 3.1e. It is observed that the GC + Au electrode has a similar behaviour that Au electrode showing two current density peaks, (one for the Au reduction at 0V and one for glycerol oxidation at 0.2V). The comparison shows that the oxidation of glycerol is possible by using Au coating on GC electrode, however, lower current densities are observed when using GC electrode as material for Au deposition than on Au electrode.

In both media, acid and alkaline, the CV behaviour of Au electrode is observed when using GC + Au electrode, furthermore, it is suggest that using GC as substrate material for Au plating has an effect in the current density, voltage shift, and the capacitive plateau during voltammetry. This effect can be due to the electrochemical potential of GC, which can affect the normal behaviour of Au electrode [25].

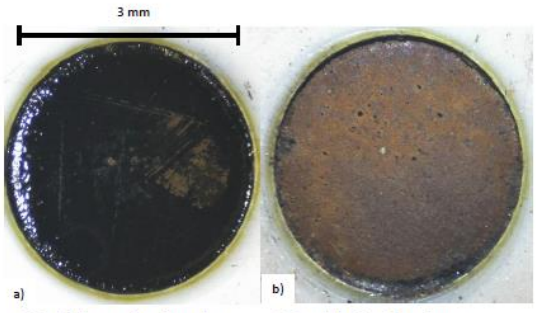


Figure 3.2 Au deposition on Glassy Carbon a) GC without Au and b) GC with Au.

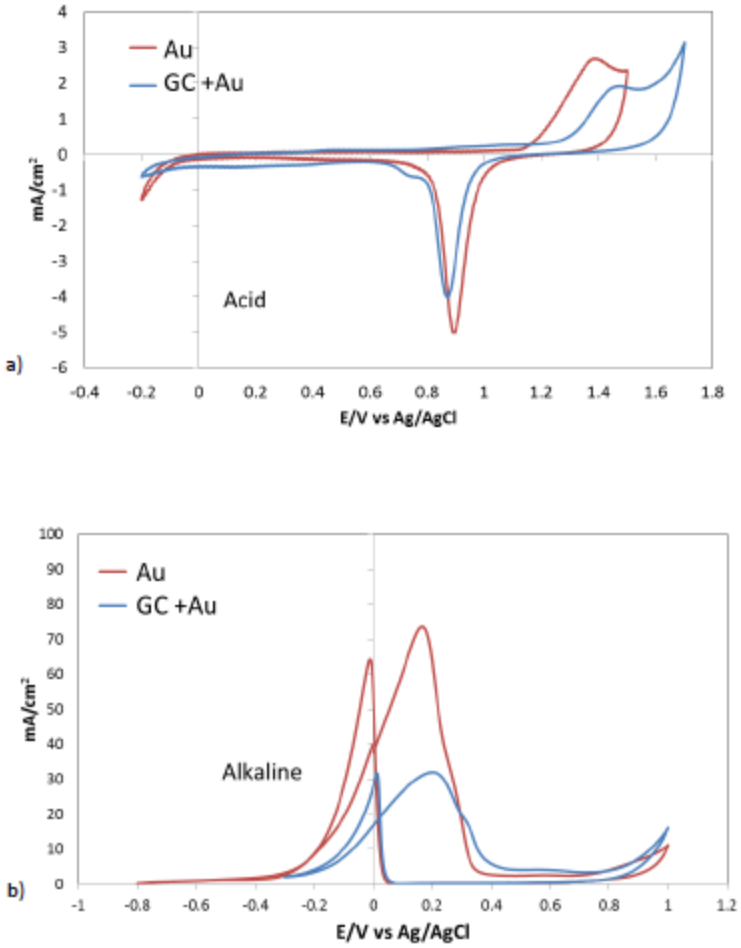


Figure 3.3 Cyclic voltammetry of coated Au electrode and GC +Au on a) Acid media (1M $C_3H_8O_3$ +1M H_2SO_4 and b) Alkaline media (1M $C_3H_8O_3$ +1M NaOH). 100mV/s and Ag-AgCl reference electrode.

3.3 Glycerol oxidation with Au catalyst on metal substrates.

The effect of metal substrates for Au catalyst coating in the glycerol oxidation reaction is shown in fig.3.4 by means of cyclic voltammetry. Different metals with Au plated are studied (Fe, Zn, Ni, Cu, carbon graphite (CG)), in order to observe the effect on current density towards anodic voltages that could favour the glycerol oxidation reaction. The metals were coated as described in section 2. The coatings were stable during all the scans when using 100mV/s and 1M $C_3H_8O_3$ + 1M NaOH electrolyte in the voltammetry window between -0.8V to 1.0V.

In general, all the substrate metal electrodes with Au coated have similar behaviour, two current density peaks are observed in all cyclic voltammetries, one corresponding to glycerol oxidation and the other to Au reduction in alkaline media. However in all the cases the current density peaks are larger and are shifted in voltage when compared with Au electrode (60mA/cm^2 at 0.2V). In detail, the CV of Fe +Au electrode shows a current density peak for the glycerol oxidation at 0.5V of 350mA/cm^2 (fig 3.4a). In the case of Zn + Au electrode (fig 3.4b), it is observed a lower current density peak at 0.4V (160mA/cm^2). CG + Au electrode shows a current density peak at 0.6V of 200mA/cm^2 (Fig 3.4c). The Ni + Au electrode has a current density peak at 0.6 of 190mA/cm^2, and the reverse scan for Au reduction occurs faster than with the other electrodes as shown in fig. 3.4d. The CV of Cu + Au electrode shows a different behaviour than the Au electrode and the other substrates, it can be observed a current density peak at -0.2V of 50mA/cm^2 and a rice in current density while the voltage is increase until 0.5V (150mA/cm^2) (Fig 3.4e). The shift in voltage and current density peak might be due to the electrochemical potential of the substrates materials [24], which can shift the oxidation of glycerol towards negative values as observed during this research.

Figure 3.5a shows the comparison of metal substrates with gold plated (Fe+Au, Zn+Au, CG+Au, Ni,Au and Cu+Au) in the voltage region of -0.5V to 0V from the cyclic voltammetry. When comparing the substrates coated with gold at a voltage of -0.2V (Fig. 3.5b) the material with the highest current density is the Cu+Au with 58mA/cm^2 and the lowest are the Ni+Au and CG+Au electrodes with 19mA/cm^2 and 18 mA/cm^2 respectively. In the case of Fe+Au and Zn+Au the max current densities observed were 25mA/cm^2 and 40mA/cm^2 accordingly.

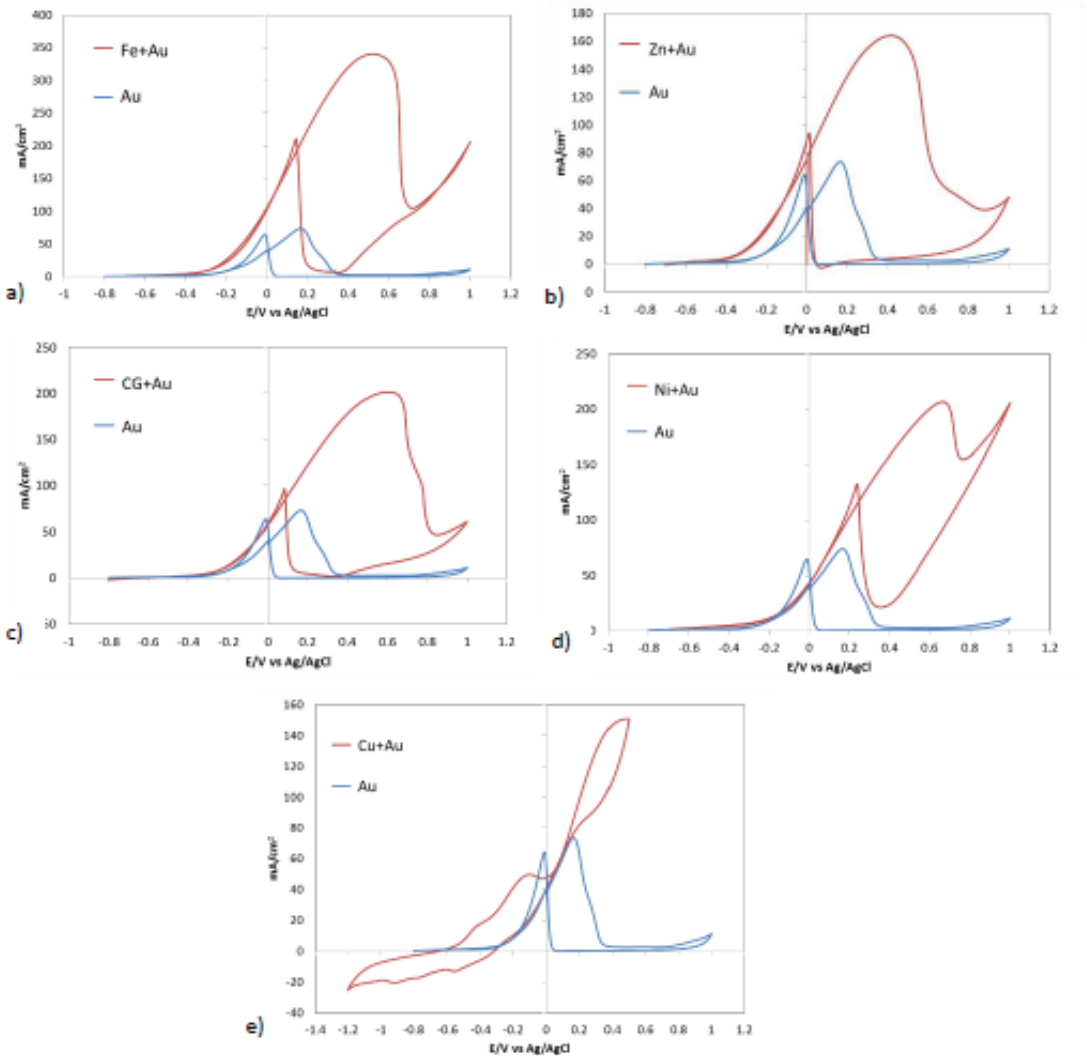


Figure 3.4 Cyclic voltammetry of a) Fe+Au, b) Zn+Au, c) CG+Au, d) Ni+Au and e) Cu+Au electrodes compare with Au electrode on 1M $C_3H_8O_3$+1M NaOH electrolyte, 100mV/s and Ag-Ag/Cl reference electrode.

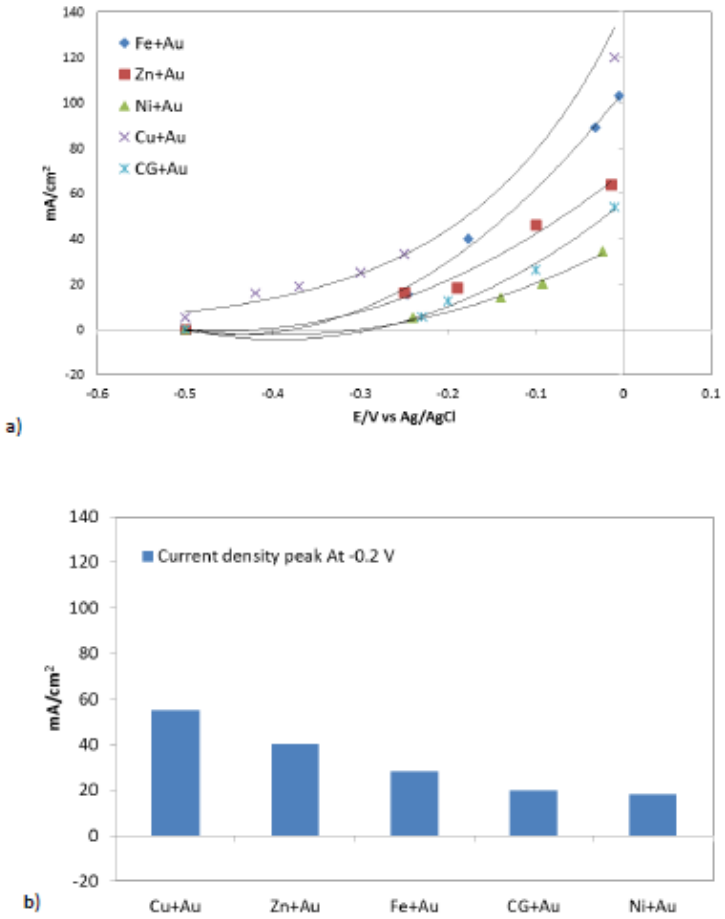


Figure 3.5 a) Comparison of Fe+Au, Zn+Au, CG+Au, Ni+Au and Cu+Au in the voltammetry region of -0.5 to 0V and b) Current density peak comparison of Fe+Au, Zn+Au, CG+Au, Ni+Au and Cu+Au at -0.2 V, on 1M $C_3H_8O_3$+1M NaOH electrolyte, 100mV/s and Ag-Ag/Cl reference electrode

3.4 Oxygen reduction reaction on alkaline media

Different commercial carbon graphite were tested using voltammetry to observe the oxygen reduction reaction (ORR) in 1M $C_3H_8O_3$ + 1M NaOH at 100mV/s and Ag-AgCl as reference electrode (the specifications of the carbon graphite can be found in section 2). The results are shown in figure 3.6.

The cyclic voltammetry of carbon graphite fibre (CGF), carbon graphite nickel (CGNi), carbon graphite K6 (CGK6), carbon graphite 90 (CG90) and carbon graphite 2765 (CG2765) are shown in figure 3.6a, 3.6b, 3.6c, 3.6d and 3.6e respectively. All the cyclic voltammetry experiments show a similar behaviour, when the voltage is increase the current density rises until the max potential set is reached (1.2V), then the back scan voltammetry begins reducing the current density until a voltage of -1.2V allowing the ORR to be observed. When comparing the carbon graphite at 1.2V: It is observed that CG2765 and CGK6 have the largest current density at the given voltage with 250mA/cm^2 and 260mA/cm2 respectively. The lowest current density found was from CG90 with 50 mA/cm^2 and CGF with 80mA/cm^2, it is also observed that in both cases CGF and CGNi have a max current density of 100mA/cm^2. A reason for the different current densities observed at carbon graphite might be due to the surface area of the carbon which can affect the conductivity of the materials as proven in previous studies [26]. Although in this study not nanoparticles or nanotubes were include in the analysis.

To analyse the ORR reaction using the carbon graphite electrodes a third order polynomial equation has been used to calculate the average of each cyclic voltammetry assuming that the capacitive behaviour of the carbon is reduce to cero. The comparison approach is shown in figure 3.7a, each cycle voltammetry is change to one single line and they are compare from -1.2V to 0.2V in the voltammetry area of each carbon graphite for the ORR effect. In all the cases current density increases when the voltage is reduce from 0.2V to -1.2V, it is observed a small difference in current densities between 0 to -0.3V in between the carbons, when the voltage is increase from -0.3V to -1.2V the difference in current density is larger, the change in current densities might due to the surface area of the carbon which is larger in the CG2765. This view is also in line with results shown in figure 3.7b in which the highest and lowest current density observed at -1.2V is from CG2765 and CG90 with 138mA/cm^2 and 10mA/cm^2 respectively.

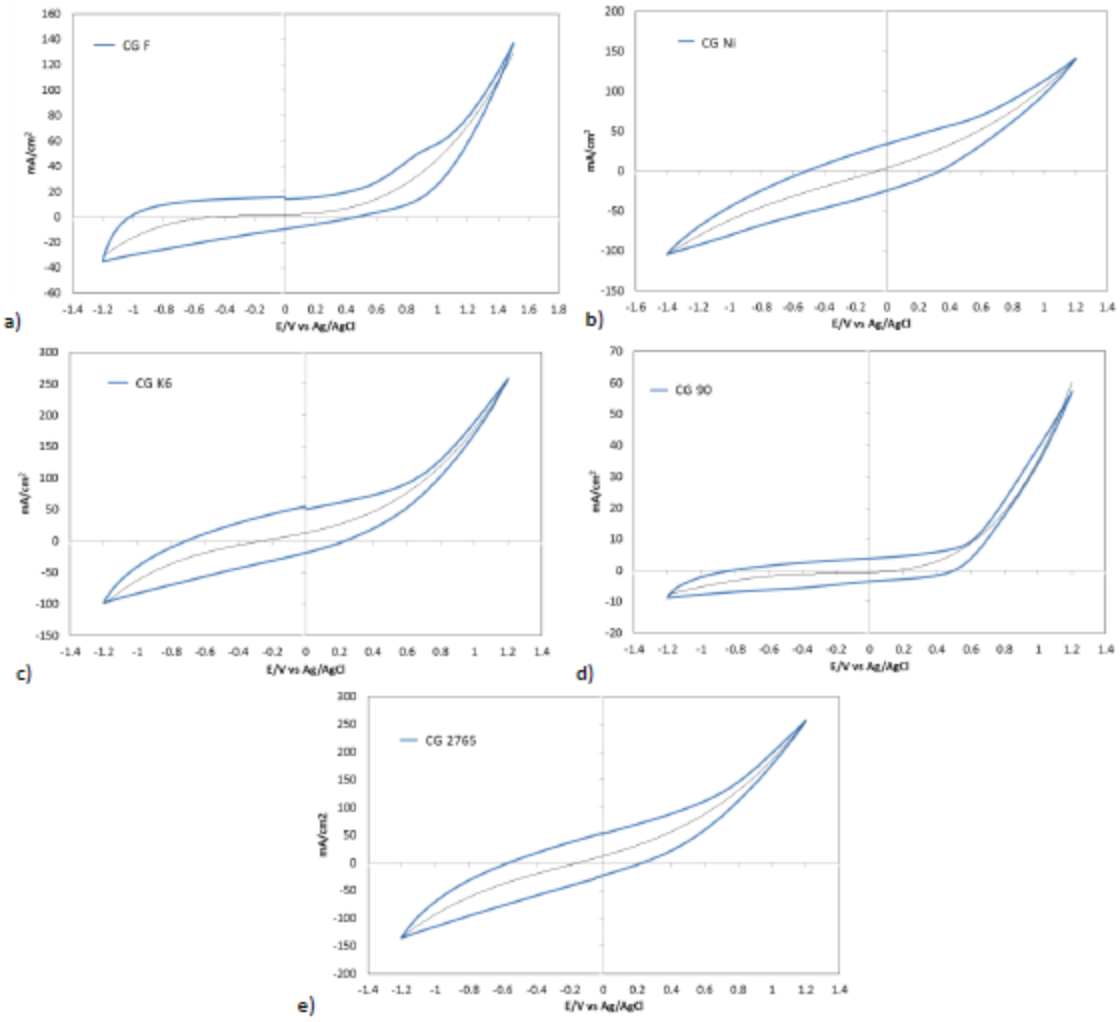
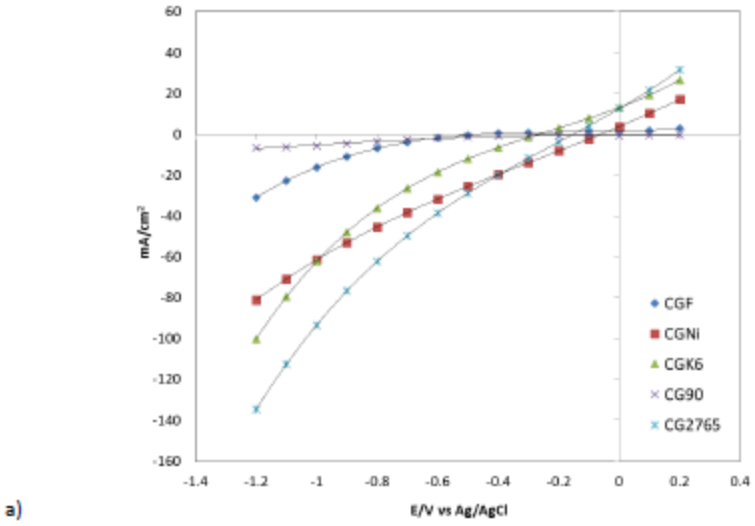


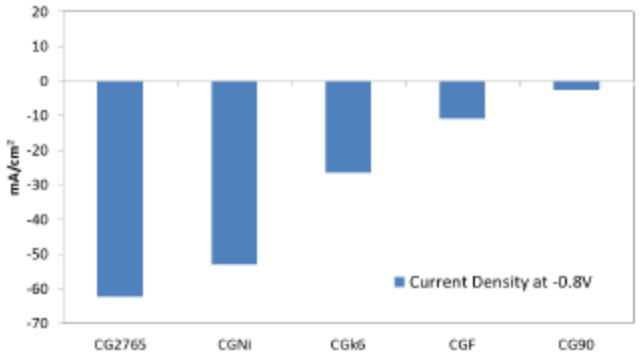
Figure 3.6 Cyclic voltammetry of: a) carbon graphite fibre (CGF), b) carbon graphite nickel (CGNi),c) carbon graphite K6 (CGK6),d) carbon graphite 90 (CG90) and e)carbon graphite 2765 (CG2765) on 1M $C_3H_8O_3$+1M NaOH electrolyte, 100mV/s and Ag-Ag/Cl reference electrode.

3.5 Discharge performance of glycerol oxidation and Oxygen Reduction Reaction

A proto type system was built to observe the discharge performance of the anode and cathode electrodes studied in this paper. Copper with electrodeposit Au was used for glycerol oxidation and carbon graphite 2765 for oxygen reduction the graphical representation of the cell is shown in figure 3.8a. The two materials were immersed in a solution of 1M $C_3H_8O_3$ + 1M NaOH electrolyte and different current density loads were connected to the cell (-0.35mA/cm^2, -0.30mA/cm^2 and -0.25mA/cm^2) and each one recorded for 300 seconds, as illustrated in figure 3.8b. When the load is increased from -0.25mA/cm^2 to -0.35mA/cm^2, the discharge voltage decreases from 0.19V to 0.15V respectively, indicating that the increase in current density reduces the voltage of the cell. Furthermore, while achieving a discharge voltages at different current densities is possible that glycerol is oxidase at the anode electrode and oxygen is reduce at the cathode electrode generating a circuit.

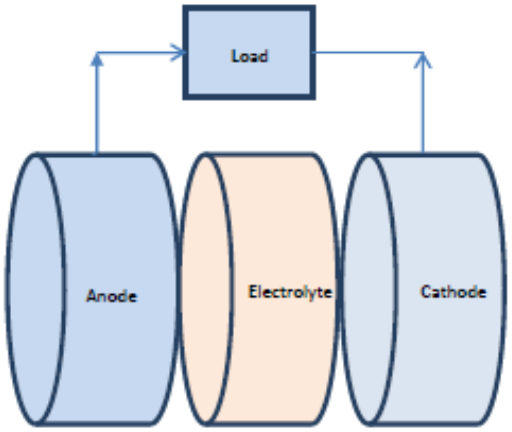


a)



b)

Figure 3.7 a) Comparison of carbon graphite fibre (CGF), carbon graphite nickel (CGNi), carbon graphite K6 (CGK6), carbon graphite 90 (CG90) and carbon graphite 2765 (CG2765) in the voltammetry region of -1.2 to 0.2V and b) Current density peak comparison at 1.2V of carbon graphite fibre (CGF), carbon graphite nickel (CGNi), carbon graphite K6 (CGK6), carbon graphite 90 (CG90) and carbon graphite 2765 (CG2765) at -0.8 V, on 1M $C_3H_8O_3$+1M NaOH electrolyte, 100mV/s and Ag-Ag/Cl reference electrode.



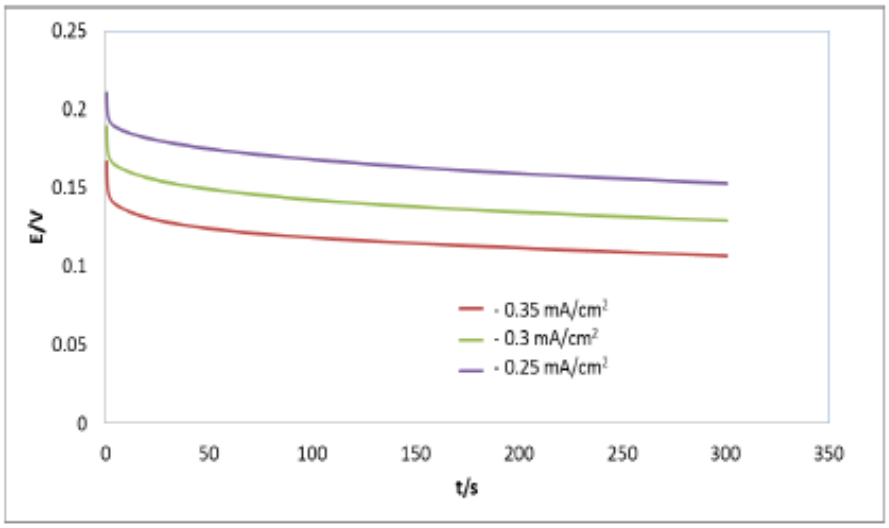


Figure 3.9 Cronoamperometry discharge experiments at -0.35mA/cm^2, -0.3mA/cm^2 and 0.25mA/cm^2 on 1M $C_3H_8O_3$ + 1M NaOH electrolytes using Zn-Au and CG2765 as cathode an anode electrodes.

4. Conclusions

It was found that Au, Pt and Cu have a higher catalytic activity towards glycerol oxidation at 1 M $C_3H_8O_3$ + 1 M NaOH than other metals, as witnessed by different current densities in the voltammetry studies. The highest current density for glycerol oxidation (10mA/cm^2) was found at -0.2V for the Au electrode. Besides, a change of substrate material for gold deposition has an effect on the current density although it deterioration was not studied yet. From the substrate materials tested (Zn, Fe, Cu, Ni and carbon graphite) it was found that copper coated with gold particles has the highest current density (of 60mA/cm^2) at a voltage of -0.2V. The oxygen reduction reaction in 1M $C_3H_8O_3$ + 1M NaOH was studied too and it was found by voltammetry that carbon graphite of different surface areas has a substantial influence on voltage favoring the ORR towards positive voltages. Carbon 2765 (Asbury) was found to have the highest current density when compared with other carbon graphite at a voltage of -0.6V (40mA/cm^2) although no nanotubes and nanoparticles were studied. An attempt to build a new simple fuel cell system for glycerol oxidation was done for the glycerol oxidation and the ORR using Cu+Au as anode and carbon 2765 as cathode materials. The maximum current density discharge that the prototype can have is -0.35mA/cm^2 in 1M glycerol + 1M NaOH

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