FP & Contract No -31588

REFLAB

"Development of a new generation biosensor for the measurement of reducing sugars in potatoes and assessment of acrylamide formation"

Instrument: Cooperative Research (CRAFT)

Final Publishable Activity Report

Period covered: 1 September 2006 – 28 February 2009
Date of preparation: 28 February 2009

Start date of project: 1 September 2006 Duration: 30 Months

Project coordinator name: **Dr. L. D. Embury**
Project coordinator organisation name: **Gwent Electronic Materials Ltd.**

Revision [draft, 1, 2, NEM V3,BZN 4]

Table of Contents

Consortium partners

SME-Partners of the project:

Gwent Electronic Materials Ltd (coordinator), UK *Gwent Electronic Materials Ltd.*	Uniscan Instruments Ltd, UK uniscan instruments
Rigas Labs SA, Greece RIGAS LABS	GTP Technology, France GTP TECHNOLOGY
Gourmet-Tiefkühlspezialitäten, Germany	Biozoon GmbH, Germany biozoon.

Non-SME partners of the project

Aviko BV, The Netherlands	Aviko

RTD-Partners of the project:

University of Nantes, France	UNIVERSITÉ DE NANTES
Wageningen University, The Netherlands	WAGENINGEN UNIVERSITY
Institute of Food Technology, Germany	Fachhochschule Weihenstephan

Section 1: Project objectives and major achievements

1.1 General project objectives and state-of-the art

The main objective of the proposed co-operative research project is to develop a low-cost system-REFLAB, which measures the reducing sugar content in potatoes and more particularly the glucose content.

The measurement of reducing sugars will be related to the potential quantity of acrylamide to be formed in relation to the thermal treatment undergone with potatoes.

The REFLAB system will be a low-cost, hand-held, robust, simple and easy to use device. The device (amperometric biosensor) is an electrochemical potentiostat capable to work at stable and changeable temperatures in connection with screen printing electrochemical cells modified with stable enzymes. The preferred configuration will be based on the association of three enzymes: Dehydrogenase/NADH oxidase/peroxidise. This configuration presents the advantage of being versatile and applicable for many other analytes by changing only the dehydrogenase enzyme and does not suffer of the oxygen competition when used as reagent-less biosensor. Another configuration based on oxidase/peroxidase can also be used but certain number of interference factors must be considered in this case. Taking into account these facts, the following technical objectives have been established (Table 2):

Table 1 Technical objectives with related work packages and milestones

Technical Objective	Deliverable/Milestone
Easy-to-use sample preparation method for enabling field analysis	D 1.1, D 2.1
Data recording possible, i.e 2 to 3 measurements per day	D 5.3, M 7.1, M 7.2
Simultaneous detection of reducing sugars (e.g glucose, sucrose)	D 5.1, D 5.2, D 5.3, M 5.1
Reduction of analysis time from a couple of hours to 5-10 minutes	D 4.2, D 6.2
Measurement range from 0.02 to 18.0 g/L	D 5.3
Correlation between sugars amount and potential amount of acrylamide to be formed	M 2.1
Cost per analysis of 2-4 Euro	D 1.1
Investment cost per device below 2.500 Euro	D 1.1
Robust and suitable for future implementation in on-line monitoring systems	D 7.1, D 7.2, D7.3

State-of-the-art

Processing techniques, the variety of potato, harvesting and storage conditions determine product characteristics (e.g. colour, flavour and texture) but also influence acrylamide levels in foodstuffs. Acrylamide is said to be a probable human carcinogen. Several studies [2,3] have revealed that acrylamide is formed as a result of the Maillard reaction between the amino acid asparagine and reducing sugars. The Maillard reaction takes place when foodstuffs are heated, and to a less extent during storage. Most of the effects of the Maillard reaction, including the caramel aromas and golden brown colours, are desirable.

In 2002 [3] high levels of acrylamide have been found in processed potato products and severe discussions in the public arouse about the health risk for the consumer, leading in the first months to a tremendous drop in sales of these products. Therefore, in the following years, the whole potato processing industry put high efforts into process improvements, looking for a compromise in order to have a product with acceptable organoleptic properties and the lowest concentration of acrylamide as reasonably achievable [1].

In experiments looking at the production of roasted potato products, it has been shown that reducing sugar levels of 1g/kg and 2g/kg in fresh weight potatoes resulted in acrylamide levels of approximately 500µg/kg and 1000µg/kg respectively [2]. It was concluded that potatoes with less than 1g/kg reducing sugar are desirable in order to retain low levels of acrylamide in roasted or fry potatoes.

Harvesting

The time at which potatoes are harvested has a significant effect on the potential for acrylamide formation. The maturity of the tubers affects the reducing sugar levels and thus, if two crops of the same variety and grown under the same conditions, but planted at different times, were harvested at the same time, the more immature potatoes would have higher levels of reducing sugar [3].

During plant growth, sucrose resulting from photosynthesis is transported to the tubers. There it breaks down into glucose and fructose, which are in turn converted into starch or are utilised for the production of energy through respiration. Sucrose is therefore a good indicator of tuber maturity during growth. If sucrose is high at harvest, the amount of glucose accumulation within the first few weeks of storage will be excessive. Once sucrose levels drop to a certain low level, the crop is ready

to be harvested and tubers should be suitable for processing if stored at the right temperature.

Storage

After harvest, sucrose in high-quality potatoes generally decreases to 1-2 mg/g and remains stable during storage, thus being no longer being a good measure of processing quality. Reducing sugars then become the indicator of process colour and acrylamide formation (see Figure 2). It has to be said that in the case of potatoes, fructose is approximately twice as efficient at promoting acrylamide formation than glucose, and 15 times more efficient than lactose [4]. Glucose levels, which were below 1 mg/g at harvest increase to a maximum after approximately 30-60 days in storage, with the extent of the increase dependent on how chemically mature the tubers were at harvest.

Sugars those become unacceptably high after harvest can be lowered in storage. “Preconditioning” is the process whereby elevated storage temperatures (about 15°C) are maintained until sugar levels drop. However, it can take several months in storage before the reducing sugars decrease to a level acceptable for processing. The length of preconditioning will depend on the sucrose content at harvest. About 70 days of preconditioning are necessary to prevent excessive glucose accumulation in tubers harvested with a sucrose level of 5-6 mg/g. If sugars are however very high at harvest, prompt marketing may be preferred. Another possibility in storage is “reconditioning”, a process whereby storage temperature is elevated before shipment of the tubers to the processor to lower again the sugar content. These elevated temperatures (up to 20 °C) allow some conversion of free sugars into starch while some are burned off in respiration.

In order to monitor the storage behaviour of potato tubers, end users need to be able to measure the content of reducing sugars over a wide range (from 0.02 to 18 g/ kg), i.e. low levels of glucose directly after harvesting (down to 0.1 g/ kg) and high levels (up to 20g/ kg) during storage monitoring and before further processing.

Measuring these reducing sugars in an industrial environment require a robust, reagent-free system, which is capable of on-site analyses and resistant against a range of interfering compounds such as Vitamin C. No cost-effective device is yet available on the market for the rapid and specific on-site parallel measurement of reducing sugars in potato storage or processing facilities. Cur-

rently, few methods are used to determine the reducing sugar levels in industrial samples (s. Table 3).

Table 2: Methods for measuring reducing sugars in food

Method	Principle	Disadvantage
Browning method	Comparison of thermally processed potatoes to a colour chart	Semi-quantitative measurement; not-objective, not GMP competitive
Benchtop analyzer	Enzymatic conversion of reducing sugars	Interference of ascorbic acid present in potatoes; expensive, no onsite application
Enzymatic test kits	Soluble enzymes convert the analyte to an indicator (mostly NAD(P)H	High personnel costs, difficult for large sample numbers
Fehling's test	Reaction based on the reduction of copper, which precipitates	Quantification difficult; interference problems
Chemical sensors	Measuring of reduction potential	Low specificity; interference of ascorbic acid present in potatoes
Test sticks	Based on glucose oxidase (GOD) system	Interference of ascorbic acid present in potatoes
Common Biosensors	Enzymatic conversion of reducing sugars	Interferences of ascorbic acid present in potatoes
HPLC	Discrimination of sugars on account of physicochemical properties	Expensive, well-trained personnel necessary; high amount of maintenance

The most common one is a bench top glucose analyzer from Yellow Springs Instruments (YSI), which is based on the detection of H_2O_2 generated by enzymatic oxidation. **All of these methods are faced with the problem of interfering substances (mainly Vitamin C), which is especially true for matrices in food and is limiting their application on real samples.** That is why chemical preparation of samples is necessary prior to measurement with any of these detection methods.
Compact hand-held biosensor device generally represents a possible solution for measuring the reducing sugars as they have several advantages. They are compact and robust in design, which makes onsite measurement possible. In addition to this, expensive measuring equipment and trained personnel become dispensable. Further advantages are the possibility to display results in real-time and to store these data, which later on can be further processed by a standard PC connection.

The biosensor to be developed in this project will have the advantages of a hand-held sensor and to be able to measure reducing sugar in real samples, combating the significant problem of electrode interference caused by ascorbic acid in real food samples.

Biosensors

Table 3: Presentation of different biosensor generations

Biosensor type	Detection method	Disadvantage
1. Generation	direct measurement of H_2O_2 at platinum electrode	highly-oxidising potential
2. Generation	combination of prior technology with electron mediators (e.g. ferrocene)	no repeated measurements possible
3. Generation	co-immobilisation of enzyme and mediator	interference from oxidations of other compounds
4. Generation (REFLAB)	**enzyme cascade system for detection both in oxidation and reduction mode**	-

Biosensors are analytical tools, which combine a biochemical recognition component with a physical transducer. The biological sensing element can be an enzyme, antibody, DNA sequence, or even a micro-organism. The biochemical component serves to catalyse selectively a reaction or facilitate a binding event. The transducer converts the biochemical event into a measurable signal, thus providing the means for detecting it. Signal measurement is based on concurrent change in a physio-chemical parameter (e.g. heat, electron transfer, light, ion or proton flow, etc.).

Different generations of biosensors have depended on the way the transducer converts the biochemical event into a measurable signal. Table 4 [5] presents an overview of the development of biosensors during the last thirty years.

A common approach to measure different analytes is to use the flavoenzyme NADH oxidase (NAox) (formular 1) as a very efficient catalyst for the oxidation of NADH, which is produced during a dehydrogenase enzyme reaction:

$$NADH + H^+ + 0_2 \Leftrightarrow NAD^+ + H_2O_2 \quad (1)$$

Measurement is based on the direct oxidation of the H_2O_2 generated in this reaction. However, these biosensors present two major disadvantages, which hinder their use in many fields of application. First, they require a potential of about 700 mV for the oxidation of H_2O_2 which leads to the problem of interference as many other compounds are oxidised under these conditions as well, leading to poor sensitivity. The second results from the necessity to add rather significant quantities of flavin-adenine dinucleotide (FAD) or flavin mononucleotide (FMN) to activate NAox.

Indeed, it has been shown that the NAox enzymes produced to date require exogenous flavin molecules for their enzymatic activity, which act as an electron acceptor [6].

The REFLAB biosensor device tackles these significant problems by developing a generic NADH-based biosensor. **The new biosensor is characterised by a low detection limit (0.02g/ L), wide range of thermostability (20 to 80 ℃) and the capacity to work both in oxidative and reductive conditions**. Depending on the matrix to be analysed and the expected concentration, the user is able to decide between an anodic or cathodic potential. This is quite significant as there may be substances in the matrix such as **Vitamin C** or phenolics, which are easily oxidised as well and thus, may significantly interfere in the measurement.

1.2 Work performed, contractors involved and the main achievements

During the 30 months of the REFALB Project the main targets were the development of redox-flexible biosensor for glucose measurement in food industries and the assessment of acrylamide formation in fried potatoes in relation with the glucose present in raw potatoes. For this achievement innovative steps have been taken:

- Production of stable enzymes needed for biosensors development
- Integration of redox-flexible biosensor concept in compact instrumental design allowing electrochemical detection at controlled temperature including an easy to use potentiostat associated with a heater-cooler system.
- Development of a reliable and simple method for acrylamide quantification

These steps have been studied in different work packages and developed according to the special end-users' needs and to the results obtained during laboratory trials.

WP 1: Specification of technical and experimental requirements

WP leader: GEM

The crucial aim of this work package was to lay down the specifications to guarantee the conformity of the final technology to the end-user's needs and to coordinate the compatibility of technical developments. In this work package, besides gathering useful information for the successful development and system integration, communication among the partners and exchange of know-how was enhanced.

A questionnaire has been prepared by GEM and completed by the end-users GOURMET and AVIKO in which their knowledge about problems in glucose measurement and accompanied interferences, together with their specifications for the final technology were summarised. This is a determinant step in order to develop a customer-driven technology.

Result presentation

End users input were considerate in collaboration with the partners in the project and a catalogue of specifications was produced. The final version of the catalogue of specifications was circulated to all partners.

WP 2: Sample preparation and correlation studies

WP leader: IFT

Based on the results of work package 1, within this work package, an easy to use sample preparation method for glucose analysis from potatoes has been developed. For this method, the potatoes have been sliced and cut to well defined square pieces according to the frying test already applied for many years in the potato processing industry. The surface glucose has been extracted by water and the glucose content has been measured by a standard method. Within 30 min the whole surface glucose has been extracted from the samples. In addition, also the glucose content of the whole potatoes has been analysed by the standard analytical method (enzyme based test kit). Correlations between the whole glucose content and the surface glucose extract have been set up to enable the calculation of the glucose content from the signal of the sensor to be developed in the future. Due to changes in the sensor design during the project, this method had been further modified in work 7 leading to the standard operating procedures for the application of the sensor device.

In addition, within the first months of the project, a quick and reliable sample extraction method for acrylamide has been developed. Based on a solid phase extraction, the acrylamide has been extracted from the potato samples by an aqueous solution, turbidity has been removed by centrifugation, acrylamide has been bound to a solid phase micro-column, eluted again by pure water and analysed by HPLC.

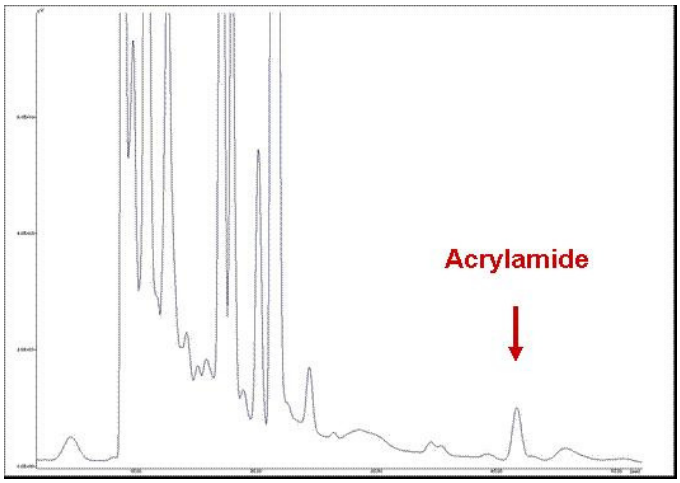


Fig. 1: HPLC chromatogram of a fried potato sample

A successful quantification of the acrylamide has been realised with a HPLC system and UV detection (Fig. 1).

In contrast to most of the analytical methods used until now for acrylamide analysis, the system has been run isocratically. Interestingly, no mass detection is needed for this system. Additionally, no organic solvent is needed as mobile phase. Acrylamide contents down to 20 µg/kg potato product have been detected with this system. Fig. 2 demonstrates the good correlation of the new HPLC method developed with the standard acrylamide analysis with mass detection for fried potato samples with different acrylamide contents (Fig. 2).

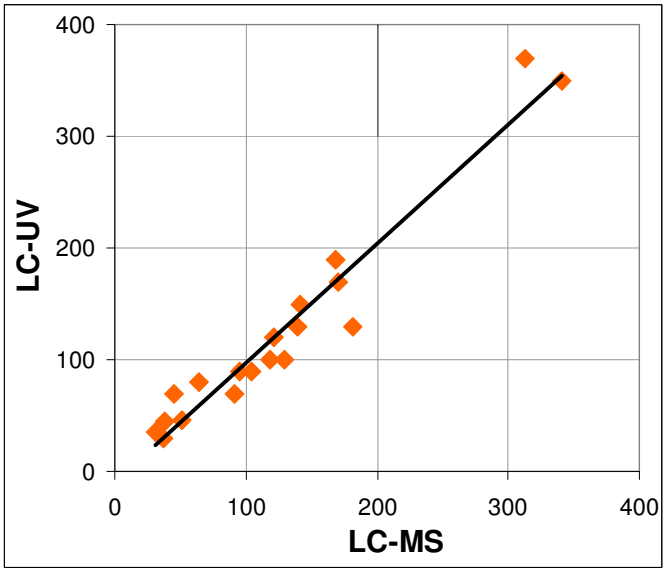


Fig. 2: Correlation of the HPLC UV (LC-UV) acrylamide detection method and the standard method with mass detection (LC-MS)

Correlations have been made between the sugar content of the potatoes and the acrylamide content of the fried product (Fig. 3) as well as the colour index used until now in the potato processing industry (Fig. 4).

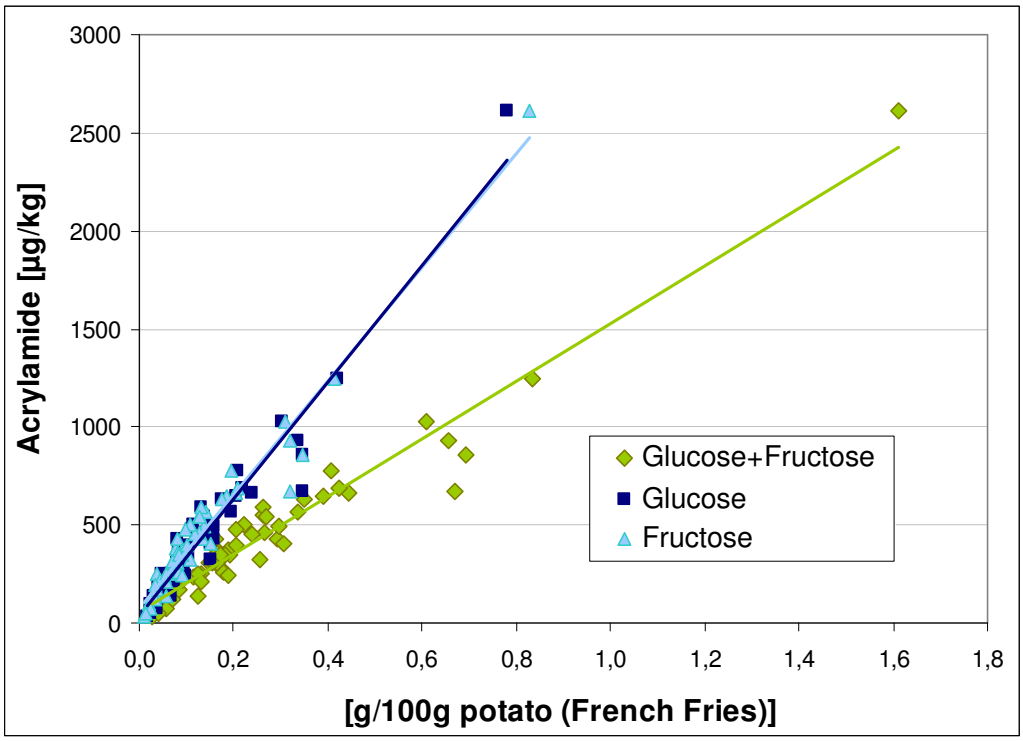


Fig.3: Correlation between sugar content (glucose, fructose, glucose + fructose) of the potatoes and the acrylamide content of the fried end product

Fig. 3 clearly indicates that there are direct correlations between glucose, fructose and the sum of glucose and fructose present in the potatoes and the acrylamide formed in the end product. Thus it has been proven, that the glucose analysis in the potatoes would be a successful tool for an acrylamide prediction in the end product.

WP 3: Isolation and characterization of thermostable enzymes

WP leader: GTP

The envisaged biosensor for measuring reducing sugars requires a set of stable enzymes. During this project, key enzymes, NADH oxidase and Glucose dehydrogenase has been cloned from thermophilic strains and characterized. Then scalable processes have been developed in order to produce, purify and store large quantity of enzymes. Moreover, a glucose isomerase (XylA) has been cloned and characterized.

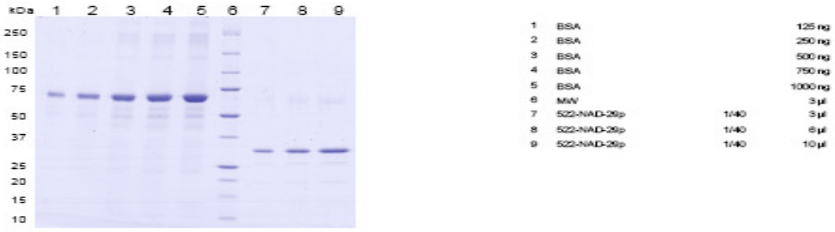
1- NADH Oxidase

The gene coding NADH oxidase from *Thermus thermophilus* (**NAoxT**) has been sub-cloned into a pGTPc expression plasmid design for the *E. coli* expression. A poly-histidine Tag has been added to the coding sequence in order to allow rapid purification and recombinant protein immuno-detection.
Another thermostable NADH oxidase has been cloned from *Archaeoglobus fulgidus* (**NAoxA**). Both enzymes have been characterized and NAoxT has been preferred over NAoxA because of its higher specific activity.
An efficient and cost effective production process has been developed for NAoxT and a pilot batch has been made [Production at 50L scale, in a steered tank fermentor, followed by purification and lyophilisation] allowed us to obtain a batch of **4.2 g of purified enzyme** [corresponding to a total of 117 500 UI (*)] with a specific activity of 29.40 U/mg^{-1} (see **Data Sheet**).

GTP TECHNOLOGY
from Gene To Protein

DATA SHEET

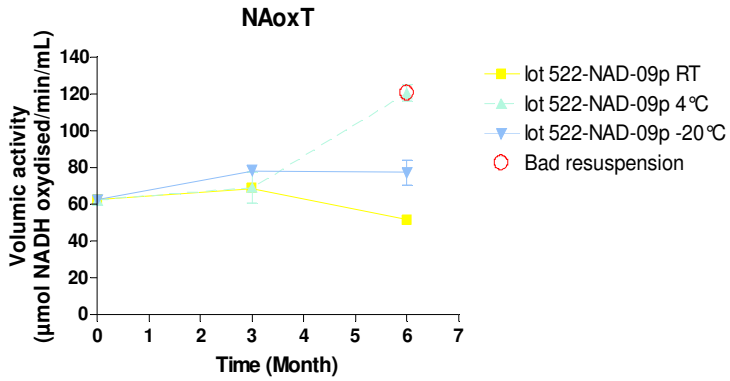
FT522-NAD-29p

Description	Recombinant NADH oxidase from *Thermus thermophilus*	**Batch**	522-NAD-29p
Origin	*E. coli*	**Concentration**	3,1 mg.ml^{-1}
Tags	His_Flag cleavable N-terminal Tags	**Volumic activity**	91,15 U.ml^{-1}
Storage	-20°C	**Specific activity**	29,40 U.mg^{-1}
Buffer	50 mM Na*PO4 pH 7,5 ; 500 mM NaCl	**Total Unit / batch**	18 230 U
Unit definition (U)	1 Unit will oxidize 1 µmol of NADH per minute at pH 7,2, at 20°C, from 100 µM NADH in the presence of 100 µM FMN	**Total lyophilised weight**	44,58 g
Number of aliquots	6 aliquots	**Equivalent volume of aliquots**	200 mL
Analysis	Each 522-NAD-29p lyophilized sample may be resuspended in an equal volume (200 ml) of buffer 50 mM Na*PO4 pH 7,5. Qualitative and quantitative analysis on acrylamide SDS-PAGE (5-18%) gel colored by Coomassie blue :  Estimation of the concentration of purified protein (densitometric analysis) : 2,6 mg.ml^{-1} Calculated apparent M.W : 30,94kDa Nadox purity estimation (densitometric analysis) : 86,52% Spectrophotometric quantitation (Bradford method) : 3,1 mg/mL		
Visa of Project Manager			

GTP TECHNOLOGY – rue Pierre et Marie Curie – BP 28262- 31682Labège cedex – France - Tel : 33 +(0)5 61 28 70 20 – Fax : 33+(0)5 61 28 70 21
S.A. au capital de 91 188 euros – APE 731Z – SIRET 433 139 557 00014
contact@gtptech.com - www.gtptech.com

(*): 1 Unit will oxidize 1 µmol of NADH per minute at pH 7.2, at 20°C, from 100 µM NADH in the presence of 100 µM FMN.

A shelf life stability study done on NAoxT shows that the lyophilized enzyme stays stable for at least 3 months whatever the storage temperature. At 6 months, the activity remains the same when the enzyme is stored at -20°C while we observed a little activity decrease at 4°C. The increase at RT is not significant; it is due to problems in lyophilized enzyme suspension.



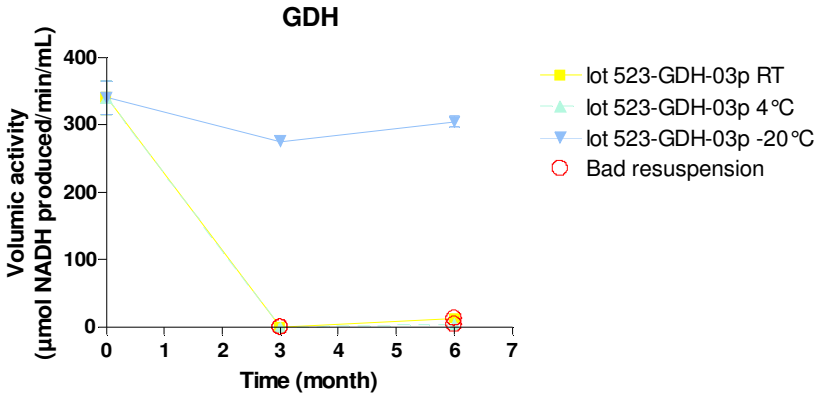
2- Glucose dehydrogenase

Based on homology to previously characterized glucose dehydrogenase, a gene (Saci_1079) was selected from *Sulfobolus acidocaldarius* genome. The gene was then cloned and the recombinant Glucose dehydrogenase (GDH) was successfully expressed in *E. coli*. The enzyme exhibited glucose DH activity with a preference for NAD as electron acceptor.

An efficient and cost effective production process has been developed for GDH and a pilot batch has been made [Production at 50L scale, in a steered tank fermentor, followed by purification and lyophilisation] allowed us to obtain a batch of **9.1 g of purified enzyme** [corresponding to a total of 117 500 UI (*)] with a specific activity of 37,7 U/mg^{-1} (see batch N° 523-GDH-20p **Data Sheet)**

Lyophilized GDH enzyme stays stable only when it is stored at -20°C.

Storage at RT or 4°C leads to a rapid and total loss of activity.



GTP TECHNOLOGY
from Gene To Protein

DATA SHEET

FT523-GDH-20p

Description	Recombinant GDH from *Sulfolobus acidocaldarius*	**Batch**	523-GDH-20p
Origin	*E. coli*	**Concentration**	3,6 mg.ml^{-1}
Tag	6xHis	**Volumic activity**	137,1 U.ml^{-1}
Storage	4°C	**Specific activity**	37,7 U.mg^{-1}
Buffer	50 mM Na*PO4 pH 7,5 ; 500 mM NaCl	**Total Unit / batch**	685,5 U
Unit definition (U)	1 Unit will produced 1 µmol of NADH per minute at pH 7,5, at 70°C, from 2 mM NAD in presence of 50 mM glucose	**Note**	Dialysed IMAC purification elution fraction (not lyophilised).
Number of aliquots	288 aliquots	**Aliquots volume**	5 mL
Analysis	Qualitative analysis on acrylamide SDS-PAGE (5-18%) gel colored by Coomassie blue :  Calculated apparent M.W : 37 kDa GDH purity estimation (densitometric analysis) : > 80 % Spectrophotometric quantitation (Bradford method) : 3,6 mg/mL		
Visa of Project Manager :			

GTP TECHNOLOGY – rue Pierre et Marie Curie – BP 28262- 31682Labège cedex – France - Tel : 33 +(0)5 61 28 70 20 – Fax : 33+(0)5 61 28 70 21
S.A. au capital de 91 188 euros – APE 731Z – SIRET 433 139 557 00014
contact@gtptech.com - www.gtptech.com

(*):1Unit will produced 1 µmol of NADH per minute at pH 7.5, at 70°C, from 2 mM NAD in presence of 50 mM glucose.

WP 4: Development of screen printed electrodes

WP leader: GEM (coordinator)

The enzymatic glucose biosensor developed during REFLAB project was designed to measure the glucose level in raw potatoes and give an indication of the estimated acrylamide level in the fried or baked product.

GEM's contribution as partner in the project was to use our expertise in order to:

- Develop materials for electrochemical sensors,
- Manufacture screen printed electrodes using these materials,
- Manufacture the enzymatic biosensor using the information and enzymes provided by partners in the project,
- Scale up the manufacture process for intermediates and final product,
- Optimise the packaging for the glucose biosensor.

Materials development for electrochemical sensors:

GEM developed suitable screen printing materials to be used for electrochemical sensors and biosensors manufacture. Using these materials we achieved a good electrochemical performance of the screen-printed electrodes and avoid interferences from materials within the useful potentials window in both oxidation and reduction modes.
The materials developed during the project are the following:

Material type	Product code	Use
Carbon ink	C2030519P4	Working electrode in electrochemical sensors
Ag/AgCl ink	C61003P7	Reference electrode in electrochemical sensors
Insulator ink	D2040917D2	Define the surface of the electrochemical sensor

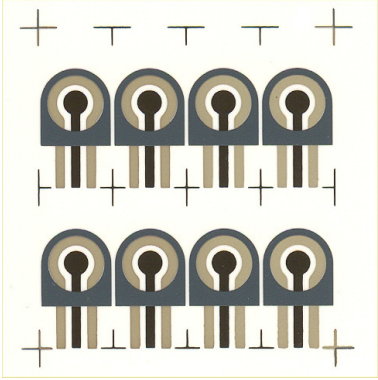
Using our internal quality control procedures and the collaboration with UNA partner we evaluated the performances of these inks and established their use for electrochemical sensors.

Manufacture screen printed electrodes:

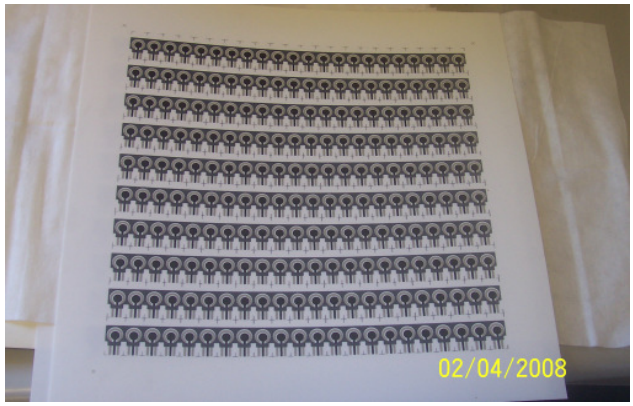
A number of sensor designs have been developed and used in order to optimise the performances of the electrochemical sensor. At GEM we have the capacity to design and redesign electrochemical sensors and produce their screen printed version.

Once the final sensor design has been agreed by partners in the project, we went to the next phase of the project: the manufacture scale up. During this stage screen printed electrodes initially produced at a scale of 8 sensors per card were redesigned in order to accommodate 220 sensors per card (see images below).

As a result of the scale up process for screen printing electrodes we were able to manufactured batches of thousands of sensors per day. The maximum capacity of sensors to be screen printed at GEM is a million and half sensors per year.



Electrode design for the feasibility study
(8 sensors per card)



Electrode design after scale up
(220 sensors per card)

Manufacture enzymatic glucose biosensor:

In parallel with developing the materials for screen printed electrodes and optimizing the design, GEM in collaboration with REFLAB partners developed and optimized the glucose biosensor using different types of enzymes.

At GEM using our specialist equipment (automatic solution dispenser –Bio-robot, temperature shelf controlled freeze dryer machine, temperature and humidity controlled packaging area), we manufactured the biosensor in different size batches, optimize the manufacturing and packaging conditions. The maximum batch size we

manufactured was 1000 biosensors per day. We also tested the biosensor for their response to different glucose concentrations using our electrochemical test facility and the protocols established by REFLAB project partners.

Conclusion:

The final glucose biosensor developed during the REFLAB project is used for glucose detection in raw potatoes in order to give an indication of the estimated amount of acrylamide that would be produced during the frying and baking processes.

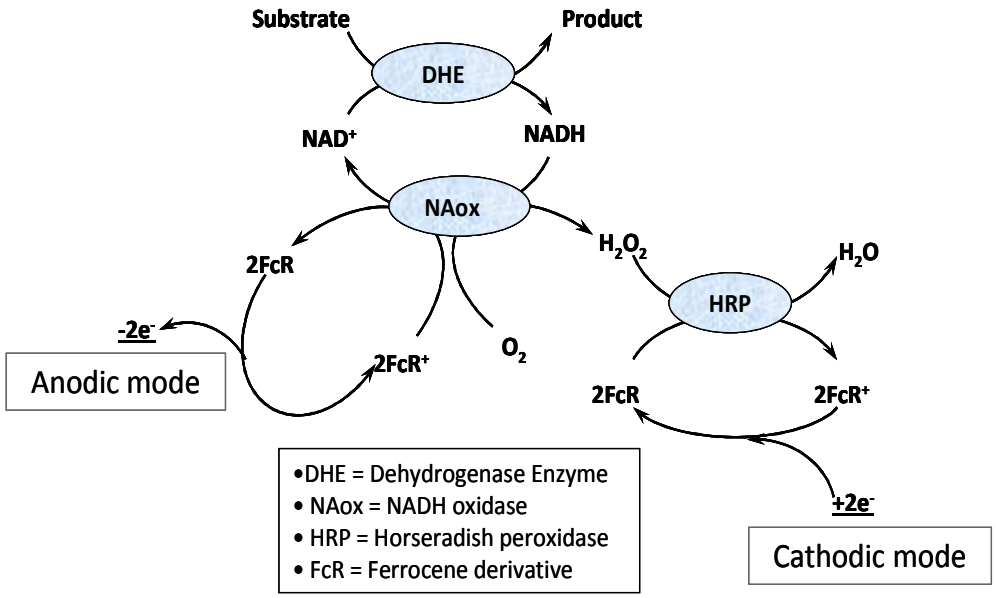
During the REFLAB project at GEM we reinforced our ability to take a product throughout feasibility studies, product development to scale up manufacturing level. At the end of the project we are able to manufacture the glucose biosensor in controlled conditions at a level of 1000 biosensors per day.

Currently, GEM in collaboration with REFLAB partners are investigating ways to promote and sell the product to potato growers and processors.

WP 5: Optimisation of biosensor components

WP leader: UNA

The concept for assays associating dehydrogenase enzymes and NADH oxidase is based on the following reaction scheme:



In REFLAB project we particularly focused on glucose dehydrogenase enzyme (GDH) with the objective to measure glucose in raw potatoes for assessment of acrylamide formation when potatoes are fried. The use of the cathodic mode presents several advantages. In particularly it allow to avoid interferences and to measure very low glucose concentrations. The biosensor working in cathodic mode presents also a large linear range. Such advantages are very valuable for the detection and quantification of glucose in potatoes especially when they are used to manufacture “French fried” or crisp potatoes. In these specific cases potatoes must contain very low glucose level so the colour of the fried material will be yellowish and not brownish and more importantly for health consideration, to avoid the acrylamide formation at high level.

- The conception and achievement of multi-enzymes biosensor was developed by University of Nantes
- The disposable transducers are screen printed type and were produced by GEM partner who also played a central part in the scale up of biosensor production.
- The enzymes GDH (glucose dehydrogenase) and NAox (NADH oxidase) are both thermo-stable and were produced by GTP partner in collaboration with Wageningen University Partner.
- The potentiostat was developed by UNISCAN partner.
- The heating-cooling assembly to function with the potentiostat was developed by RIGAS LABS partner.
- Tests and validations were conducted by the following partners: GEM, University of Nantes, Institute of Food Technology (Weihenstephan), Biozoon, Aviko and Gourmet.

A redox-flexible glucose biosensor devoted for food analysis and more particularly for very low glucose measurement in potatoes was achieved. Its analytical behaviours were optimized in order to reach industrial needs (2% repeatability, <3% accuracy, > 1 year shelf life stability, 20 seconds response time and 3 minutes for the whole measurement time, very simple and quick sample preparation …).

The validation was conducted by comparing the results obtained with the biosensor to those obtained for the same solutions and in the same analytical conditions by the spectroscopic standard method.

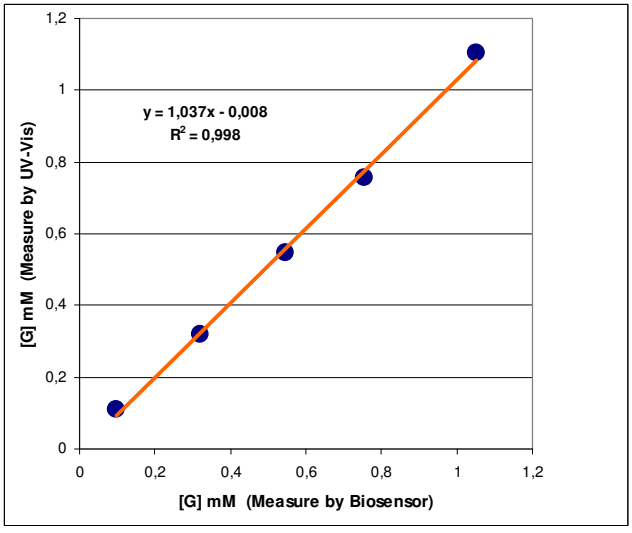


Figure: Biosensor validation (glucose solutions) with UV-Vis standard method (Enzymatic Kit).

Special attention was paid to the adaptation of the biosensor for measuring glucose in real samples of potatoes. The problem of interference, very frequent in the case of amperometric biosensor, was specifically considered and excellent results in this field have been obtained.

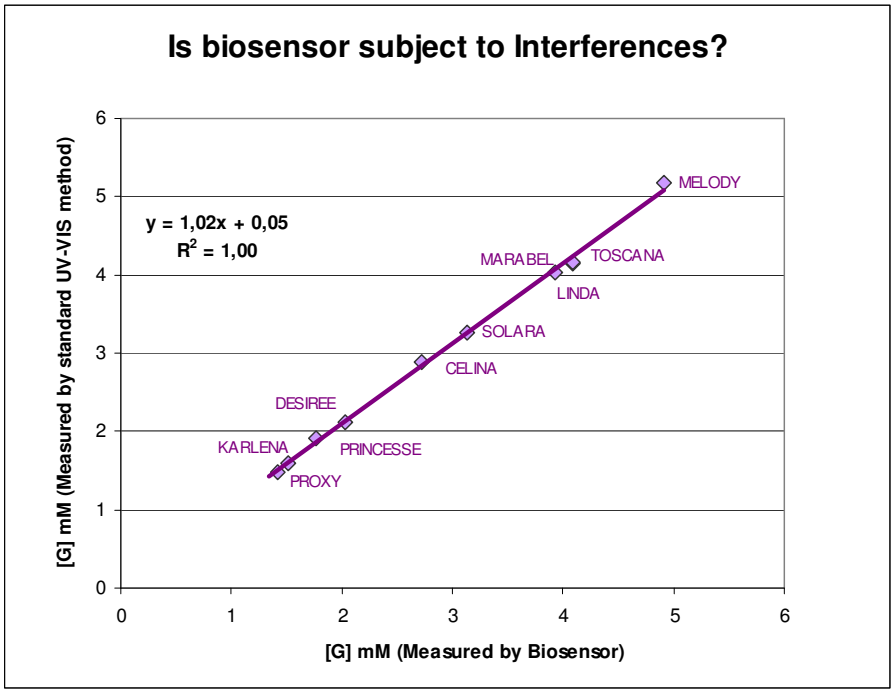


Figure: Validation of the glucose sensor with different potato samples

WP 6: Development of the electronic interface and data recording system

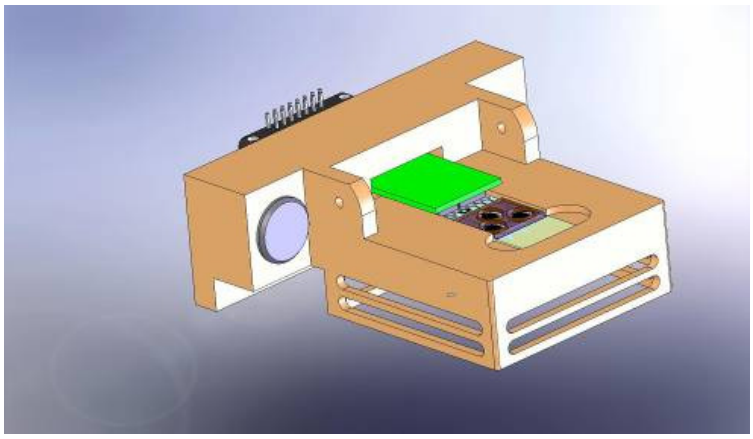
WP leader: UNISCAN

Within this work package the following development were achieved:

- Lab measurements and electronic circuit simulations have been performed to assess the feasibility of adding a multi Working Electrode (WE) multiplexing system and heater control to a UNISCAN PG580R potentiostat.
- The software which runs on the potentiostat was modified to run the REFLAB experiment standalone. It controls all aspects of the experiment and controls the heater assembly. It walks the user thought the measurement in an ergonomic and logical procedure.
- A heater-cooler unit was developed for which the result description (product(s) envisaged, functional description, main advantages, innovations) are described below:

A heating-cooling assembly in conjunction with the potentiostat was developed for the reliable operation of the Biosensor technology. The completed sensor is required to operate at a constant temperature for providing reproducible measurements of reducing sugars in potatoes. The required temperature of the sensor will be determined by the sensor developers.

The principle of the temperature control is based on a Peltier device. This is a device, which has the capability to heat or cool depending on the polarity of the electrical current passing through it. This facilitates accurate temperature control. The design is based on housing the Peltier device as a stand-alone unit connected with the potentiostat with a slot for the sensor. The electronic control for the Peltier is based on PID technology and is housed in an electronic module adjacent to the potentiostat. A software application is provided for remote controlling the heater/cooler assembly through the serial port or USB port. Temperature setting point can be sent to the instrument at any time, taking into consideration a valid temperature range of 15 to 50^{o}C.



Engineering Design of Heater/Cooler Assembly

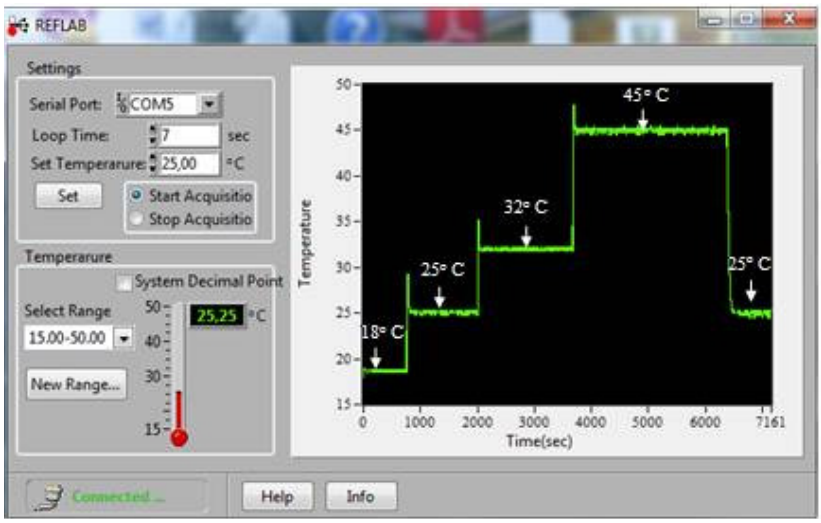
Possible market applications (sectors, type of use ..) or how they might be used in further research (including expected timings)

A heating-cooling device for temperature control of screen printed electrodes is not commercially available in the market. Market applications of the heater/cooler system in the area of analytical chemistry with emphasis in electrochemistry include whenever constant temperature is needed using thermostable enzyme systems for measuring various analytes.

Stage of development (laboratory prototype, demonstrator, industrial product...)

Three prototypes of the heater cooler were developed in conjunction with the potentiostat.

One heater/cooler prototype as a stand-alone instrument to be interfaced with a potentiostat



Software Control of REFLAB Heater/Cooler

A final version of the heater/cooler assembly is under development including either local keypad control or PC software control with a goal to be used with various potentiostats.

Collaboration sought or offered (manufacturing agreement, financial support or investment, information exchange, training, consultancy, other**)**

Collaboration with various companies which manufacture potentiostats will be sought in order to interface our heater/cooler assembly. In addition collaboration with University of Nantes is established in using the heater/cooler assembly in screen printed electrodes with various enzyme chemistries and potentiostats.

WP 7: System integration and validation

WP leader: IFT

Within this work package, 2 major objectives have been reached. These are the development of standard operating procedures for the sensor device and the validation of the whole system within field trials with potato samples coming from industrial productions.

a) Standard Operating Procedures:

Based on the former work packages and within the system integration; Standard Operation Procedures (SOP) for the whole system developed have to be defined. These SOP's are divided into 4 different steps: The sampling of a representative sample taken from a storage or a truck, the sample pretreatment, the sensor measurement and the forecast of the acrylamide content based on the sensor signal. For all these steps successful SOP's have been defined that will be further validated during the field trials. In a first step, 20 potatoes will representatively be collected. From each potato, a well defined strip will be cut. These strips will then be mashed in a standard blender, the sample solution will be filtrated and a small amount is put on the sensor. The sensor signal will then be correlated to an acrylamide content expected for the relevant process parameters and the sugar content.

b) Validation trials

After the implementation of the new control electronic, the temperature control and the innovative sensor development in one device, the different components have been tested for a validation step by step to receive a clear view of the reliability of the different components especially in field trials taking potato samples for the potato processors present in the consortium.

From these validation trials it may be concluded, that the new 3 electrodes sensors may be controlled by the potentiostat and control software and the temperature control unit integrated in this system. Validation trials with potato samples taken directly from the potato processing clearly indicate, that the biosensor developed is running with a high precision even in these real samples with repeatability of less than 2 % and an accuracy in the same range. The fact, that no influences of interferences

have been observed even for the samples taken from the production processes had been very important for the applicability of the sensor device in the industrial field (see results of Work Package 5).

Correlation studies done for the forecast of the acrylamide formation during the frying process demonstrated several interesting new facts (Figure).

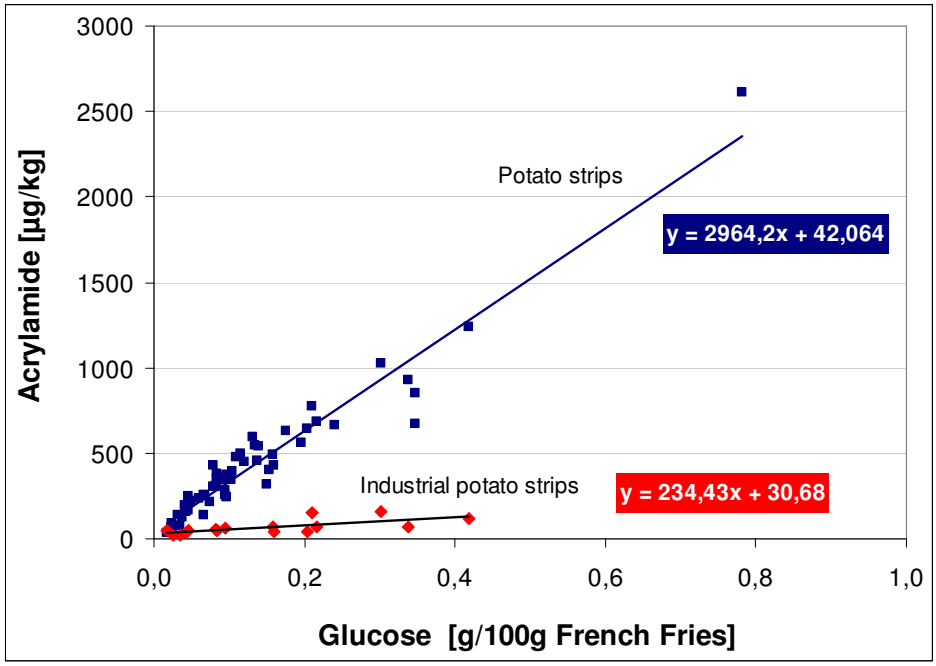


Figure: Correlation between glucose content and acrylamide formation in the end product for home made potato strips (blue) and industrial produced strips (red)

For the application of home made fried potatoes a direct correlation of the glucose content and the acrylamide content has been noticed. This fact has also been found for the potato strips processed for the production of deep fried French fries that had been treated with different washing and blanching steps prior to the pre-frying and deep frying. But due to these pretreatments, the acrylamide content in the fried end product had been less than 10 % of the home made fried product made directly from the untreated raw potatoes. As a consequence, different correlation graphs have to be taken for the industrial processed and home made fried potato products to get a reliable forecast of the acryamide content formed in the end product.

WP 8: Dissemination and pre-exploitation measures

WP leader: BZN

The aims are to guarantee a proper dissemination of the project results to the desired target audiences and to set up from the very early stage of the project the basis for IPR protection and future exploitation activities.

A project flyer has been prepared by GEM containing general information about the REFLAB project, mainly regarding the objectives and the project consortium description and contacts. This has been a valuable dissemination tool used by the partners. The flyer and and direct e-mailing will continue to be the main force for contacting future customers in higher education, research and industry.
Another very important meas of dissemination is the project website put togehter by the Coordinator.

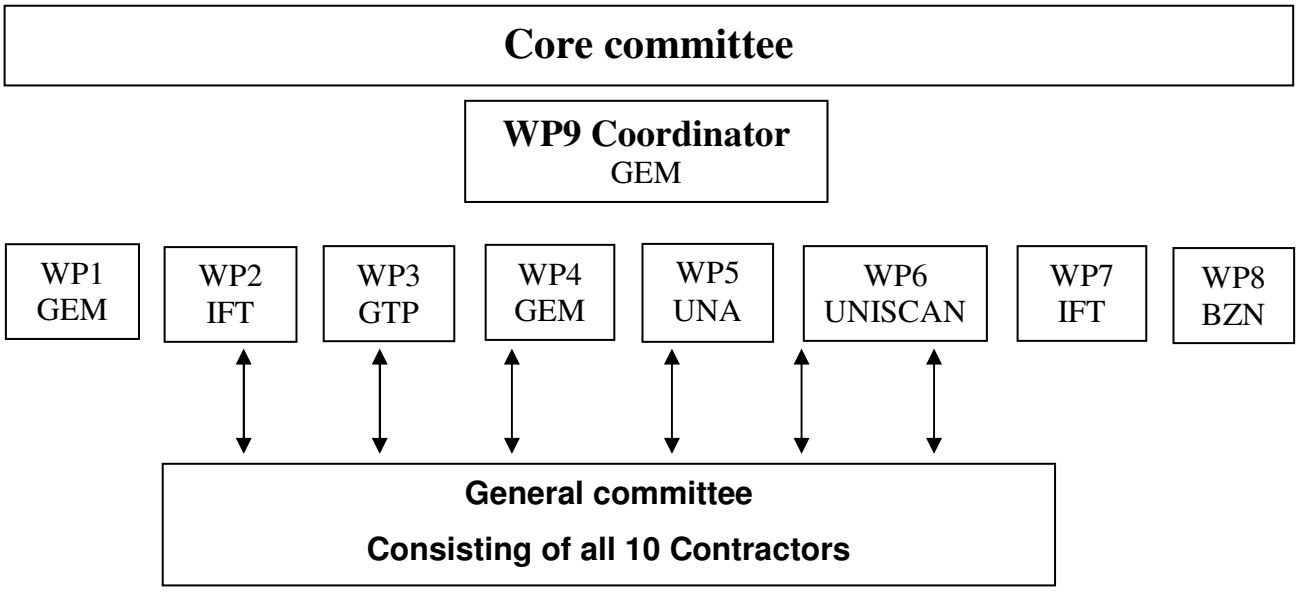
Other worth to mention dissemination actions were taken, such as:

1. Attendance/ presentations at relevant trade fairs/ conferences /seminars / workshops
2. Direct contacts to relevant industries, organisations and R&D centres
3. Demonstrations by the end users

WP 9: Project management

The project management of the REFLAB project included structural, informative, organisational and coordination tasks, as well as intercultural and communicative functions.

As seen in the following chart the project team consists of a Core Committee (inclu ing the coordinator and the Work Package Leaders) and a General Committee (in-cluding all project members). All members are responsible for the smooth and uhampered work flow throughout the project.



During the entire duration of the project a continuous communication flow has been maintained among the members of the Consortium. Contribution of every partner to the overall consecution of the project, discussions among partner team of subtasks and team leaders of the tasks and up-dating of the general situation of different work to be executed have intensively been kept. Fluent information share has been provided by meetings, telephone, e-mailing and post. GEM, as general coordinator responsible of the overall management of the project, supported by BZN, has been responsible of collecting all relevant results derived from the tasks leaders, meeting preparations, partner communication, and other administrative and financial issues as well as the scientific/ technical and IPR management.

Section 2 – Dissemination and use

Partner WU presented a Poster Communication at the Annual Protein Meeting of the NWO-CW study group in Lunteren (10-12 December 2007) entitled:

> "Purification and Characterization of glucose dehydrogenase from the thermoacidophilic archaeon Sulfolobus acidocaldarius" by Ratnesh Kumar Singh, Marco Siemerink, Servé Kengen, John van der Oost. Presented at the annual Protein meeting of the NWO-CW study group in Lunteren,

Partner UNA also held various poster and oral presentations at International Scientific Meetings:

1. *Poster Communication:* " Redox Flexible Biosensors: Tools for Accurate Detection of Very Low Substrate Concentration in Complex Matrices"08 - 12/09/2008 - International Society of Electrochemistry – Seville (Spain)
2. *Oral Communication:* "Kinetic Studies to Better Comprehend the Electron Transfer, via Mediators, between Redox Enzymes and Electrode Surface and hence to Dispose of Interferences" 08 – 12/09/2008 – International Society of Electrochemistry – Seville (Spain)
3. *Oral Communication:* " Biocapteurs ampérométriques "redox- flexible" à usage unique : Avantages et inconvénients des mesures en mode anodique ou cathodique" *(Planned)* 06 – 10/07/2009 - Journées d'Électrochimie JE09 – Sinaia (Romania)
4. *Oral Communication:* " Biocapteurs ampérométriques "redox- flexible" à usage unique : Novelles avancées pour la compréhension des cinétiques impliquées" *(Planned)* 06 – 10/07/2009- Journées d'Électrochimie JE09 – Sinaia (Romania)
5. *Poster Communication:* " Disposable Biosensors and Bioassays: Use of Redox-flexible system for detection of low substrate concentrations" *(Planned)* 16 – 21/08/2009- ISE 60th Annual Meeting: From Single Biomolecule Electrochemistry to Biosensors and Biofuel Cells – Beijing (China)

Partner UNA talked about REFLAB Project and the scientific results in several Plenary and Invited Lecturers at International Scientific Meetings:

1. *Plenary Lecture:* "Redox-flexible dehydrogenase biosensors: Original platform for analytical tools to be used in biology, food and environment". 18 – 20/07/2007 Balkan Environnemental Association & International Conference of Alba Iulia 2007. Alba Iulia, (Roumania).
2. *Invited Lecture:* "Doped carbon paste as material to improve electron transfer reactions of amperometric biosensors" 09 - 14/09/2007 – International Society of Electrochemistry – Banff (Canada)
3. *Invited Lecture:* "Biosensors as Analytical Tools for the Evaluation of the Quality and Safety of Food, Water, and Environment" 15 – 17/05/2008 - Coreach Sustainability Conference – Beijing (China)
4. *Plenary Lecture:* "Redox Flexible Biosensors and their Use in Food Analysis"09 – 11/10/2008 - International Conference of Industrial Microbiology and Applied Biotechnology – Galati (Romania)
5. *Invited Lecture:* "Disposable Biosensors and Bioassays: Kinetic considerations for the implementation of a generic biosensing platform" *(Planned)* 16 – 21/08/2009 - ISE 60th Annual Meeting: From Single Biomolecule Electrochemistry to Biosensors and Biofuel Cells – Beijing (China)

Several Publications are planned for the very near future.

Partners IFT and WU are individually preparing some articles for publication:

1. N. Polner, T. Loetzbeyer: Development of a cheap sensitive method for acrylamide detection in fried potato products based on HPLC, Potato Research
2. N. Polner, T. Loetzbeyer: Acrylamide reduction during industrial processing in fried potato products, Potato Research
3. “Purification and Characterization of a glucose dehydrogenase from the thermoacidophilic archaeon Sulfolobus acidocaldarius” by Ratnesh Kumar Singh, Marco Siemerink, Servé Kengen, John van der Oost.
4. “Production and characterization of a thermostable xylose isomerases from the thermophilic bacterium Fervidobacterium gondwanense”, by Ratnesh Kumar Singh, Leon Kluskens, Servé Kengen, John van der Oost

The Coordinator, GEM has also presented the Reflab Project at several eventy, such as:

Biosensor 2008 was an international conference organised by Elsevier on 14-16 May 2008 in Shanghai, China.
Web site: http://www.biosensors-congress.elsevier.com/
The conference covered most of the biosensors' related thematics: immunosensors, enzymatic biosensors, DNA sensors etc. as well as commercial developments, manufacturers and markets, new signal transduction technology, etc. The event consisted in plenary lectures, oral presentations, posters, exhibitions and presenters from 52 counties attended the conference. GEM had an exhibition stand and the REFLAB flyer was presented to the audience.

Electrochem 2008 was an international conference organised by Royal Society of Chemistry on 15-17September 2008 in Liverpool, U.K. Web site: http://www.rsc.org/ConferencesAndEvents/conference/alldetails.cfm?evid=101263
The conference covered thematic related to electrochemical sensors and biosensors including the enzyme base type biosensors. The event consisted in plenary lectures, oral presentations, posters, exhibitions and presenters from 10 counties attended the conference. REFLAB flyer was presented to the audience during this event.

During the last 2 years Partner UNISCAN has attended several events where they presented the Reflab Project:

1. Electrochem 2007, 2008 London 2007, Liverpool 2008, UK.
2. Biosensors 2008, May 2008 Beijing
3. Regional Electrochemistry meeting of South East Asia, Singapore, August 2008
4. International workshop on biosensors, Goa, India, October 2008
5. International Society for Electrochemistry, Edinburgh 2007, Banff, Canada 2008
6. Microscience 2008, London, June 2008.

References

[1] Opinion of the Scientific Committee on Food on new findings regarding the presaence of acrylamide in food, 2002

[2] Biedermann-Brem. S.; Noti, A.; Grob, K. Imhof, D.; Bazzocco, D. and Pfefferle, A., (2003) How much reducing sugars may potatoes contain to avoid excessive acrylamide formation during roasting and baking? Eur. Food. Res. Technology 217, 369-373.

[3] Putz, B. (1993) The sugars of the potato as a subject of tuber-physiology. European Association for Potato research: Abstracts of the 12th Triennal Conference, Paris, 1993, 244-246

[4] Biedermann, M.; Noti, A.; Bierdermann-Brem, S.; Mozzetti, V. And Grob, K. (2002) Experiments on acrylamide formation and possibilities to decrease the potential of acrylamide formation in potatoes, Mitteilungen aus Lebensmitteluntersuchung und Hygiene 93, 668-687

[5] Vreeke, M. S. IVD Technology Magazine Archive 07/1997

[6] Mizutani, F.; Sato, Y.; Hirata, Y.; Sawaguchi, T.; Yabuki, S. Sens. Actuat. B-Chem. 2000, 65, 46-48.