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Deliverable Leader:	TU Wien
Contributors:	Andreas Schwaighofer, Georg Ramer, Bernhard Lendl (TUW), Julia Kuligowski (HULAFE), Jennifer Karrer (QRT)
Reviewers:	ALPES
Approved by:	ALPEs

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Executive Summary

This deliverable discusses the general makeup of human breast milk with a special focus on protein composition. Several approaches for analysis of proteins in milk are specified and compared. Subsequently, IR spectroscopy for analysis of proteins is introduced, and the advantages of Laser-based IR transmission setups for analysis of milk are clarified. The setup employed for method development for the prototype is presented and the IR spectra of target proteins casein, α-lactalbumin and lactoferrin are discussed. Ultrasonication is necessary as pretreatment of the breast milk prior to IR analysis and preliminary measurements of the optimization of the process parameters are shown. Finally, future steps for successful method development are outlined.

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Definitions, Acronyms and Abbreviations

Acronym	Title
PLS	partial least squares
EC-QCL	external cavity – quantum cascade laser
SNR	signal-to-noise ratio
MCT	Mercury Cadmium Telluride
FTIR	Fourier transform infrared

1. Requirements for human milk analysis

1.1. End users' feedback

A questionnaire (see appendix 1) was sent out to several pediatricians but no direct reply was received. In general, the respondents suggested, that an analyzer for milk protein composition could be a tool of interest for further research.
Nutrishield partner HULAFE is a potential end user for the Nutrishield human milk sensor and has contributed largely to the selection of the analytes hereafter.

1.2. Medical impact of the makers of choice

Proteins in human milk can be classified into the whey, casein and mucin fractions, which each contain several proteins. The contents of casein and whey proteins in breast milk change profoundly early in lactation. In the beginning, the concentration of whey proteins is very high, whereas almost no casein is detectable during the first days of lactation. As a consequence, there is no fixed ratio of whey to casein in human milk. The frequently cited ratio of 60:40 is an approximation of the ratio during the course of lactation, but it does vary from 80:20 in early lactation to 50:50 in late lactation. Because the amino acid compositions of caseins and whey proteins differ, the amino acid content of human milk also varies during lactation (Lönnerdal, 2003). In comparison, cow, sheep and goat milk has a casein-to-whey protein ratio of 80:20. Mucins are present at low concentrations and located in the milk fat globule membrane. The most abundant proteins are caseins (2.2 g/L), α-lactalbumin (2.2 g/L), lactoferrin (1.9 g/L), secretory IgA (1.1 g/L).
The individual proteins fulfill important nutritional and immunological tasks in the newborns metabolism.

- It was found that β-caseins are capable of complexing calcium ions, thus facilitating their absorption. κ-caseins were reported to inhibit the activity of diverse bacteria.
- α-lactalbumin binds calcium and zinc ions and it is assumed that this protein has a positive effect on mineral absorption, possibly by the generation of peptides that facilitate the absorption of divalent cations. Furthermore, it has been found that α-lactalbumin has antimicrobial activity against multiple bacteria.
- Lactoferrin binds a major portion of iron in human milk and facilitates the uptake of iron in the presence of a specific receptor. Clinical trials with bovine lactoferrin added to infant formula have not shown any enhancing effect on iron absorption or iron status, which may be because bovine lactoferrin does not bind to the human lactoferrin receptor. Furthermore, this protein has also shown antimicrobial activity.
- Secretory IgA and other immunoglobins can improve the immunity of the newborn and even transfer mother's immunity against several general pathogens to her breastfed infant in the form of sIgA (Lönnerdal, 2003).

There are multiple factors that influence the amount and composition of proteins in breast milk. For example, the protein content of milk obtained from mothers who deliver preterm is significantly higher than that of mothers who deliver at term. Subsequently, protein levels decrease in human milk over the first

4 to 6 weeks or more regardless of timing of delivery. Furthermore, the protein content increases with maternal body weight, and decreases in mothers producing higher amounts of milk.
Several studies have been performed to investigate the variability and importance of individual protein concentrations influenced by diverse factors.

- For example, temporal changes of major proteins in preterm and term milk have been investigated. It was found that preterm human milk at the same postmenstrual ages (39 -48 weeks) have α-lactalbumin contents significantly lower than term human milk. This study reveals limited availability of α-lactalbumin, a nutritionally important protein, in breastfed preterm infants and suggest importance of this point for optimization of human milk fortification (Garcia-Rodenas et al., 2018).
- A different study found that the freezing/heating/pasteurization processes applied to human donor milk prior to delivery to neonates could affect the concentrations of lactoferrin, secretory IgA and lysozyme (Chang et al., 2013).
- A systematic literature review study was performed to identify the correlation of the lactoferrin concentration in breast milk and lactation. Here, studies investigating ten maternal factors (ethnicity, age, parity, socioeconomic status, nutritional status, smoking, mastitis, various maternal infections) and 2 infant factors (infection, prematurity) on the lactoferrin concentration were summarized and compared. It was found, that particularly the lactation stage correlates with lactoferrin concentration, colostrum has the highest lactoferrin concentration, and it decreases significantly with days postpartum. It was stated that a limitation of the comparability of this review was the lack of a standardized and fast method for determination of the lactoferrin concentration (Villavicencio et al., 2016).
- A study found that the concentration of sIgA quickly decreases during the first weeks of lactation. This variability during lactation reflects the important role of human milk in supplying immunological factors in the first days of life (Ballabio et al., 2007).

In summary, for feeding preterm infants, the lower level of total protein and specific proteins from donor (typically, term, late lactation) milk alone is limiting, and requires additional supplementation (Ballard and Morrow, 2013; Andreas et al., 2015). For formulation of this supplementation as well as the processing of human donor milk, knowledge of the protein composition in breast milk is of major importance.

1.3. Detailed description of the human milk sample

Human milk is a complex biological liquid composed of thousands of constituents. It contains an aqueous phase with true solutions (87%), colloidal dispersions of casein molecules (0.3%), emulsions of fat globules (4%), fat-globule membranes and live cells. The main constituents include proteins, non-protein nitrogen, carbohydrates, lipids, vitamins, minerals and cells. The composition of human milk varies among women and is influenced by genetic individuality, maternal nutrition, stage of gestation and lactation. Fat is the most variable constituent in human milk, e.g. the milk fat content is associated with weight of the mother, the total milk volume and increases during a single nursing (Picciano, 2001; Verd et al., 2018).
The mean macronutrient composition of human milk is estimated to be approximately 9-12 g/L for protein, 32-36 g/L for fat and 67-78 g/L for lactose, and energy values varying around 650-700 g/L, depending on the fluctuating fat content. Compared to milk from other species that are employed in human nutrition, human milk contains lower levels of proteins (cow: 34 g/L, goat: 2.9 g/L, sheep: 55 g/L), average levels of fat (cow: 37 g/L, goat: 45 g/L, sheep: 74 g/L), and high levels of lactose (cow: 46 g/L, goat: 41 g/L, sheep:

48 g/L). These differences can be explained by the different needs of the offspring at very young age, for example cows need higher protein to grow quickly, as they descend from prey animals in the wild and need to be able to run at an early age. In contrast, humans milk contains high levels of sugars as they need specific sugars and also fats to nourish the growth of the brain, spinal cord and nerves. Consequently, milk from other species need to be altered first to more closely resemble human milk (Jenson, 1995; Ballard and Morrow, 2013).
In summary, breastfeeding and human milk are the standards for infant feeding that provide the optimal nutritional mix for infants in the first six months (exclusive breastfeeding), with continuation for two years and longer as complimentary food.

2. Proposed Nutrishield Sensor

2.1. Laser-based mid-IR Spectroscopy

Mid-IR spectroscopy is a well-established analytical technique routinely employed to study the structure of polypeptides and proteins in a label free manner. Vibrations of the polypeptide repeating units of proteins result in nine characteristic group frequencies in the mid-IR region referred to as amide bands. For investigation of the protein secondary structure, the amide I (1600-1700 cm^{-1}) and amide II band (1500-1600 cm^{-1}) is most commonly used (Fabian and Mäntele, 2006).
When working with conventional FTIR spectroscopy, the low feasible path lengths for transmission measurements of proteins in aqueous solution are a considerable impairment for the robustness of analysis and impede flow-through measurements and high-throughput applications. This is particularly the case for liquids with high viscosity and complex matrix composition, such as milk, that features a diverse chemical makeup involving fat, protein and lactose (Aernouts et al., 2011). When milk is inserted into a liquid cell with low optical paths, it tends to clog or form air bubbles, thus preventing robust sample handling. This experimental constraint arises from the combination of two aspects: first, the HOH-bending band of water near 1645 cm^{-1} with a high molar absorption coefficient, which overlaps with the protein amide I band; and second, the low emission power provided by conventional thermal light sources (globars) that are used in FTIR spectrometers. As a consequence, most commonly employed path lengths for IR transmission measurements of proteins in aqueous solutions are below 10 µm to avoid total IR absorption in the region of the HOH-bending vibration (Fabian and Mäntele, 2006).
As an alternative light source for mid-IR measurements, quantum cascade lasers (QCLs) have been introduced two decades ago (Faist et al., 1994). They provide spectral power densities several orders of magnitude higher than thermal light (Weida and Yee, 2011), rendering these light sources highly interesting for transmission measurements of aqueous samples (Kolhed et al., 2002; Lendl et al., 2000). Since then, custom-built setups based on QCLs have gained numerous implementations in mid-IR spectroscopy in process analytical applications as well as in biomedical spectroscopy of gaseous and liquid samples (Schwaighofer et al., 2017). In IR transmission spectroscopy of proteins, the transmission paths could be considerably increased by using an external cavity-quantum cascade laser (EC-QCL) light source that provides significantly higher emission powers (Alcaráz et al., 2015). Laser-based IR transmission measurements were successfully performed for examination of the protein secondary structure by evaluation of the amide I band (Schwaighofer et al., 2016; Alcaráz et al., 2016). Further, the viability of

protein discrimination and quantitation in commercial bovine milk samples was successfully demonstrated (Kuligowski et al., 2017; Schwaighofer et al., 2018a). Most recently, a new and improved EC-QCL based IR transmission setup was introduced for analysis of the protein amid I and amide II regions, allowing more sensitive quantitative and more detailed qualitative analysis of proteins (Schwaighofer et al., 2018b).
Figure 1A depicts a schematic of the setup for analysis of the protein amide I and amide II bands. As a light source, a latest generation EC-QCL was employed featuring a spectral emission range of approx. 260 cm^{-1} that covers the amide I and amide II bands of proteins. Furthermore, the setup comprises a temperature-stabilized sample cell and a Peltier-cooled MCT detector. With this setup, it is possible to work with a transmission path length of 31 µm that ensures robust sample handling and high-throughput measurements. Figure 1B shows IR spectra of the amide I and amide II regions of the relevant proteins casein, α-lactalbumin and lactoferrin. Even though the proteins feature relatively similar band maxima originating from α-helical secondary structure, initial evaluations have shown that concentration of individual proteins in ternary mixtures can be achieved by partial least squares (PLS) regression analysis.

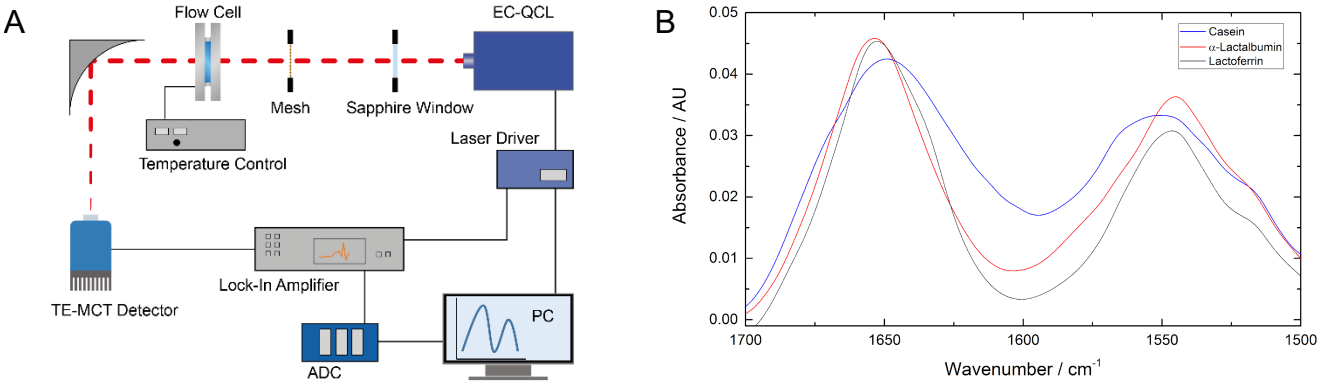

Figure 1: *(A) Experimental setup for laser-based IR transmission setup for analysis of the protein amide I and amide II bands. (B) IR spectrum recorded with the laser-based IR transmission setup of the proteins casein, α-lactalbumin and lactoferrin.*

2.1.1. Specifications

For the Nutrishield sensor using mid-IR laser spectroscopy for quantifying the protein composition of human milk either a discrete frequency (DF-IR) approach or a tuning laser-based approach are considered. In DF-IR in a first step a small number of single wavelengths emitting at the most significant wavelengths for an analytical problem are selected. This allows to perform measurements in the sensor using potentially cheaper lasers without moving parts. However, in case of overlapped bands the number of required wavelengths is higher, making a tunable mid-IR source, such as an external cavity – quantum cascade laser (EC-QCL) a more efficient solution. The type of light source used for the human milk sensor will be decided by the stake holders of this problem in Nutrishield: TUW, QRT and ALPES.
The sensor will be designed as a transmission-absorption experiment using CaF_2 windows with an optical pathlength of ~30 µm. In order to improve the sensitivity of the sensor a balanced detection scheme can be evaluated. Here, the laser beam is split into two before the sample. Only one of these beams passes through the sample. The intensity of both is measured on matched detectors. This serves to reject fluctuations of the laser beam intensity.

For evaluation of the quantitative performance of a laser based optical system in terms of signal-to-noise ratio (SNR), practical requirements need to be defined. In practical applications of IR spectroscopy in liquids (Schwaighofer et al., 2018b), this is usually performed by assessing the performance of the entire setup in the way as described in the following. The two crucial values for evaluation of the SNR are (i) the noise floor of the setup and (ii) the interaction length with the sample, e.g. the optical path in transmission measurements. Assessment of the noise floor is performed by calculation of 100% lines. After setting up the setup, i.e. light source, sample cell filled with solvent and detector, several single beam spectra are recorded and then the absorbance is calculated. Under ideal conditions, the result would be a flat line at 100% transmittance, corresponding to zero absorbance. However, due to several noise contributors in the system, the resulting absorbance spectrum is not flat but contains the noise floor of the setup. By evaluation of the RMS (root-mean-square) of the 100% line, the noise floor is quantified. The second important value in this context is the "signal" originating from a certain analyte concentration. For evaluation, single beam spectra are recorded from an analyte at known concentrations and the absorbance is calculated with the solvent as a reference. The height of the absorbance measured at a given wavenumber is the analytical signal, which is proportional to the path length employed, according the Beer-Lambert Law. From these numbers, the SNR can be calculated for a given analyte, which is wavelength dependent. Consequently, this is not a useful result, in case multiple analytes at diverse wavenumbers are considered.
For this reason, a practical and meaningful approach for assessing the SNR is to specify the noise floor of the setup in terms of a 100% line and the interaction length of the sample interface. For the EC-QCL based IR transmission setup that was used to record the calibration data set for milk analysis as outlined in the previous section, the RMS noise of the 100% line was ~5.10^{-5} absorbance units when employing a transmission cell with a path length of 31 µm. With these values total protein, casein and β-lactoglobulin content could be successfully quantified by PLS with the quality parameters outlined in Table 1. In single wavelength spectroscopy, the noise contributions are different than in broadband spectroscopy and need to be individually evaluated.
Regarding the implementation of the optimum optical transmission path length, this parameter relies on the detectivity and dynamic range of the detector, the emission power of the employed laser as well as the selected wavelength for analysis. For analysis of proteins, measurements of wavelength in the amide II region can be performed at higher optical transmission path (~factor 5) than for measurements of the amide I region, due to the overlap of the latter band with the intense HOH-bending band in this spectral region. The optical transmission path employed in the Nutrishield prototype for protein analysis in breast milk will be empirically optimized. Table 1, summarized the key measurement and component parameters of the Nutrishield prototype to be developed and a comparison with the reference benchtop EC-QCL transmission setup.

Table 1: *Key measurement and component parameters of the nutrishield prototype.*

	Commercial EC-QCL setup	Nutrishield prototype for breast milk analysis
Laser emission power [mW]	5 - 10	up to 50 mW
Sample interaction length [µm]	31	to be optimised

Detector detectivity [cm $Hz^{0.5}$ W^{-1}]	1.6 x 10^{10}	4 x 10^{8}
Mode of operation	pulsed	pulsed
Duty cycle [%]	50	0.5 – 50?

2.1.2. Advantages compared to standard analytical techniques

For analysis of individual proteins in human milk, there exist multiple methods based on sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Velonà et al., 1999), automated microfluidic SDS-PAGE (Affolter et al., 2016), high performance liquid chromatography with ultra violet detection (HPLC-UV) (Ferreira, 2007), enzyme-linked immunosorbent assay (ELISA) (Chang et al., 2013), among others. These techniques rely on wet-chemical sample preparation (dilution, precipitation, separation, setting pH,…), thus they are labor-intensive and the throughput is rather low.
Furthermore, there exists a commercially available instrument based on Fourier transform infrared (FTIR) spectroscopy, the human milk analyser (HMA) (https://www.mirissolutions.com/) by Miris AB (Upsala, Sweden). By analysis of the mid-IR spectral region with an optical path length of 50 µm, the HMA gives the energy, fat, carbohydrate and total protein content in milk. The analysis requires at least 3 mL of sample and takes 60 seconds time. Here, a homogenization step is needed as sample preparation.

2.1.3. Sample preparation

Before the IR measurement, the raw breast milk needs to be homogenized, in order to break up the fat globules to decrease the average fat globule diameter and to narrow the distribution. This procedure increases the accuracy of results and decreases light scattering by large fat globules. Furthermore, large fat globules can also lead to the Christiansen light-scattering which causes a change of in the refraction of light at wavelengths near maximum absorption by the carbonyl and carbon-hydrogen groups. The Christiansen effect causes a shift in the apparent wavelength of maximum light absorption to a longer wavelength. This effect can be reduced by decreasing the fat globule diameter. (Di Marzo et al., 2016). It was suggested that homogenization prior to IR transmission measurements should reduce fat globules diameters to less than 2 µm (Remillard et al., 1993).
Within the Nutrishield project, homogenization of raw breast milk was optimized by using a laboratory scale Sonicator (Branson Digital Sonifier 450) equipped with a microtip horn. The optimum parameters to achieve maximum homogenization at mildest conditions were found to be at output level 5 (~55 W) at a duty cycle of 50 % with a runtime of 40 cycles (50 sec). Using these conditions, the temperature of milk increases from initial 22°C to 44°C. At this temperature, the milk fat is fully melted (Wang et al., 2017). The progression of homogenization at different parameters was monitored using Laser Light Scattering (LLS) and microscopy (Michalski et al., 2001). Figure 2 shows LLS measurements performed by a Mastersizer 2000 (Malvern Instruments, Malvern, UK). The untreated milk (red line) displays a broad particle size distribution with a center at 6 µm. Figure 3A shows a microscope image of this milk sample,

indicating large fat droplets. As further shown in Figure 2, with increasing sonication times, the size distribution significantly narrows and the center shifts to particle sizes of approximately 1 µm.
The corresponding microscope image of the homogenized sample (Figure 3B) qualitatively confirms the results obtained by LLS and demonstrates successful disintegration of the fat globules and no presence of large fat globules any more.

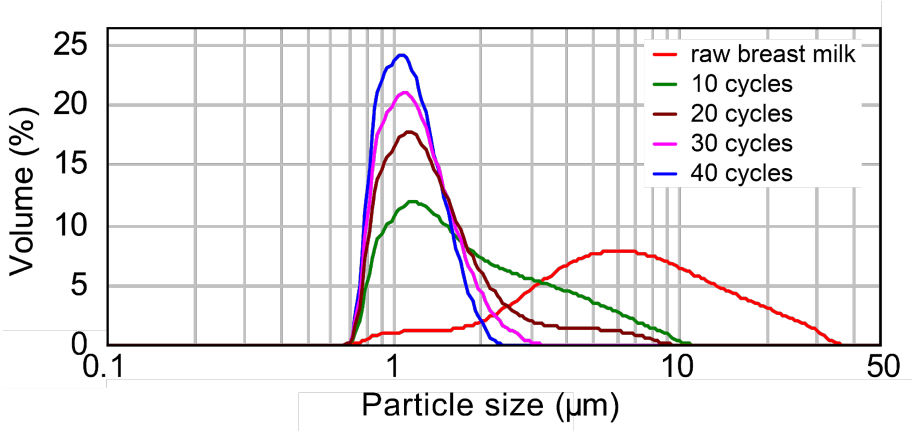

Figure 2: *Particle size distribution obtained by LLS of human milk samples that were ultrasonically treated for different periods of time. The red line indicates untreated breast milk. Green, red, pink and blue lines show results of breast milk that was homogenized for 10, 20, 30 and 40 cycles.*

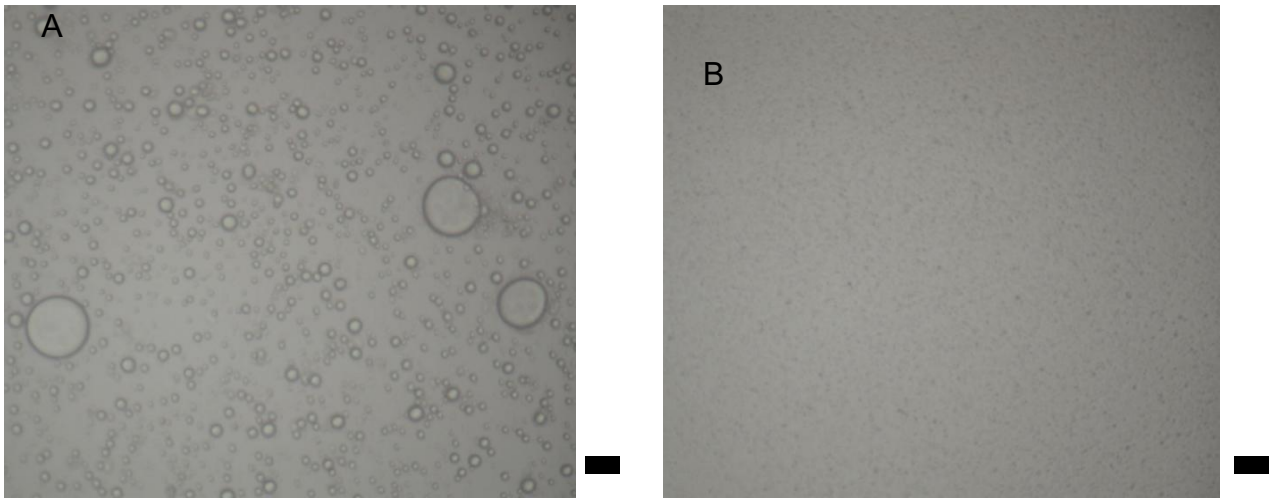

Figure 3: *Microscope image of (A) untreated breast milk sample and (B) breast milk sample that was homogenized for 40 cycles. The scale bar indicates 20 µm.*

2.2. Reference analytics

Within the Nutrishield project, for total protein analysis the HMA Miris will be employed. Commercial ELISA kits for the determination of casein, lactoferrin and alpha-lactalbumin will be employed for quantification of individual proteins in human milk samples.

2.3. Downstream work plan

In order to improve the sensitivity (LOD/SNR) for detection of protein composition of human milk, a balanced detection design will be evaluated by TUW. The advantage of balanced detection noise suppression will be weighed against its larger cost and complexity together with QRT before implementation of the Nutrishield sensor.
Using the lab setup for protein quantification at TUW a PLS model for protein in human milk will be established. Here, reference samples and measurements will be provided by HULAFE.

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4. Appendix

4.1. Questionnaire

Questionnaire

Requirements for human milk analysis

Editor:
Jennifer Karrer (QRT)

Contributor:
Julia Kuligowski (HULAFE)
Georg Ramer (TUW)

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1. Introduction

1.1. Mission of NUTRISHIELD

NUTRISHIELD, short for "Fact-based personalized nutrition for the young", has the goal to build a mobile and interactive platform for guiding EU citizens towards personalized nutritional plans. It contributes to reducing diet-related health disorders. NUTRISHIELD will investigate mid-IR sensing solutions to analyse relevant parameters for nutrition.

1.2. Motivation

There is a general expectation from society that food will be safe. However, what can be considered as "safe" for many people may not be for a few others. Each individual responds differently to the same food or nutrient. This is determined by genetic factors as well as by acquired factors, such as the metabolic and physiological states, the development of the microbiome, dietary habits, the amount of stress and exercise in daily life. In recent decades, the range of food choices available to consumers greatly expanded as well as the number of healthy food choice oriented-services. However, the number of people suffering from health problems related to unhealthy nutritional behavior in the EU remains on the rise, while well-intentioned generic advice on healthy food choices may confuse, and in some cases even mislead, consumers. The need for an efficient and reliable platform to personalize nutrition, based on the acquisition and scientific interpretation of genetic and acquired factors, is today evident.

1.3. Concept

NUTRISHIELD aims at creating an innovative framework to support personalized nutrition based on a comprehensive set of genetic and environmental factors. The approach will be validated through three clinical studies. In order to bring analytical capabilities to a larger number of practicing physicians, NUTRISHIELD will investigate different analyzer concepts based on the Quantum Cascade Lasers technology. Leveraging both existing and newly acquired knowledge on personalized nutrition, NUTRISHIELD will finally develop a mobile application to support individual personalized food choice, building on existing apps Platemate© and Carbcounter©, developed by the NUTRISHIELD consortium.

1.4. Aim of this questionnaire

The human milk and urine analyser are considered as potential applications. Molecules or biomarkers in human milk, which are important for its quality control shall be determined. On the other hand, urine analysis requirements are collected.

2. Interviewee characterization

2.1. Please state the type of industry or organization you are representing:
(e.g. hospital, research institute, milk bank, etc.)

2.2. Please fill your name and state your position in the organization:

2.3. Please state your view on urine and human milk analysis with the help of the following questions:

To the best of your knowledge, what is the current standard method used for human milk analysis in the industry you represent?

Are you monitoring human milk in your organization/industry?

What and how are you measuring it?

How often are you measuring these parameters?

What is the usual sample volume?

How long does it take to measure these analytes?

Is there a standard you are following if you measure these analytes? If yes, which one?

<u>If you have a device, which measures human milk:</u>
What is the name of the device/method? Are you satisfied with it and why?

How many devices do you have in your organization?

How much is one device?

3. Human milk analysis requirements

3.1. What analytes/analyte groups should be measured with the novel monitoring device? Why?

3.2. What is the measurement range of interest of the desired group of analytes/analyte you want to measure?

3.3. What do you think about analysing human milk for the following analytes:

Analyte	very useful	probably useful	not required	Comment
Fat				
Carbohydrate				
total protein				
Casein				
Lactoferrin				
α-Lactalbumin				
trans fats				
ns A, B1, B2, B3, B6, B12, C, D				
ree amino acids				
ligosaccharides				
FAS (ω-6 linoleic acid series & ω-3 linolenic acid series; DHA, AA, EPA)				

3.4. Do you know other people, with interest/expertise in human milk? How are they analysing it? Would you be willing to give us the contact information?

3.5. Is there a need to investigate the influence of different conditions on human milk?

	need	interested	need and interest	Comment
Pasteurization				
Storage				
Enrichment				

3.6. Technial Aspects (ONLY for the most needed/useful analytes)

Analyte 1:	
LOD	
Working range (up to)	

Response time (how much delay is acceptable after taking the sample?)	
Measuring interval/data gathering frequency	
Analyte 2:	
LOD	
Working range (up to)	
Response time (how much delay is acceptable after taking the sample?)	
Measuring interval/data gathering frequency	

Are there any technical restrictions for the instrument?

Parameter	**Target value / Description of optimum case**
Max. Calibration frequency	
Max. Maintenance frequency	
Max. warm-up time after setup/reset in min	
Restrictions on cooling/heating requirements	
Weight (max. acceptable (in kg))	
Dimensions (max. acceptable LxWxH (mm))	
Power Limitations (are there any power limitations (e.g. max. watt consumption, grid independency, resilience against power outage etc.))	
Further limitations (e.g. medical certification)	
Regulations, laws, standards in force Are there any applicable regulatory restrictions?	
Restrictions on chemical solvents type	
Disposal of used solvent	
Disposal of contaminated sample stream	

What kind of data output would be required?