

Application of advanced light microscopic techniques to gain deeper insights into cheese matrix physico-chemistry

Zuzana Burdikova¹ · Zdenek Svindrych² ·
Cian Hickey¹ · Martin G. Wilkinson⁴ ·
Mark A. E. Auty¹ · Ota Samek³ · Silvia Bernatova³ ·
Vladislav Krzyzanek³ · Ammasi Periasamy² ·
Jeremiah J. Sheehan¹

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Abstract Despite considerable advances in confocal microscopy and related technologies, advanced light microscopy techniques have been rarely used to study the microstructure of cheese matrices. Here, we demonstrate the potential of advanced microscopic techniques, namely fluorescence lifetime imaging (FLIM), confocal Raman spectroscopy and non-linear microscopy (two-photon excitation and second harmonic generation microscopy), to study localized physicochemical properties of cheese matrices. These methods allow for precise localization of individual chemical components and help to determine their spatial organization in three dimensions. We discuss both fluorescent labelling and label-free methods that provide valuable information about the localized environment. Overall, these new technologies will enable a greater understanding of the influence of manufacturing processes on cheese quality and consistency.

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✉ Zuzana Burdikova
zuzana.burdikova@teagasc.ie

✉ Jeremiah J. Sheehan
diarmuid.sheehan@teagasc.ie

¹ Teagasc Food Research Centre, Moorepark, Fermoy, Co., Cork, Ireland

² Department of Biology, University of Virginia, Charlottesville, VA, USA

³ VUT, Brno, Czech Republic

⁴ Department of Life Sciences, University of Limerick, Limerick, Ireland

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1 Introduction

Cheese is a complex soft matter composed of a protein matrix entrapping fat, water, minerals and dispersed bacterial colonies and their metabolites. The characteristic structure, texture and flavour properties of cheese result from a sequence of dynamic physico-chemical, microbial and biochemical processes starting in the cheese vat during manufacture and continuing throughout ripening and cold storage. The textural and rheological properties of a cheese are dependent on interactions between its structural units, including those between fat globules and the protein matrix, fat globules coated with casein micelles which may interact with cheese matrix, moisture content, pools of free fat, levels of proteolysis and casein matrix bond strength (Everett and Auty 2008; Hickey et al. 2015).

Fat may be entrapped in the cheese matrix as inert filler or as a copolymer within the casein matrix. It can exist in globular form, consisting of droplets ranging in size from 0.2 to 15 μm with an average of 4 μm (Lopez et al. 2007) surrounded by milk fat globular membrane material (MFGM) or it may exist as non-globular free fat. The MFGM is a layer of surface active material which envelops the fat globule and is composed of proteins, enzymes, glycoprotein, triacylglycerols, phospholipids, glycolipids, cholesterol and other minor components (Lopez and Briard-Bion 2007; Hickey et al. 2015). Certain cheese manufacture processes such as high cook temperatures result in coalescence of fat globules along with fracturing of the MFGM during the ensuing pressing of the curds results in the formation of free fat and the release of MFGM material (Lopez and Briard-Bion 2007; Hickey et al. 2015). Amphipolar properties of the phospholipids in the MFGM also enable protein-fat interactions which are essential for matrix structure and enable fat globules to act as emulsifiers within the matrix stabilizing both oil-in-water and water-in-oil emulsions. This is probably responsible for bacterial colonies within the cheese matrix locating at the protein-fat interface in contact with the MFGM due to the hydrophobic nature of LAB bacteria (Banks 1991; Lopez et al. 2006; Pereira et al. 2009; Gallier et al. 2010).

Calcium and phosphorus are the key mineral components within cheese matrices and participate in the protein network that forms through milk coagulation and have a major effect on the structure and texture of cheese (Upreti and Metzger 2006; Lucey and Fox 2015). Increased levels of acidification during cheese manufacture results in conversion of colloidal calcium phosphate to the soluble form which is lost in the whey (Sheehan and Guinee 2004). Excess calcium in the cheese post manufacture can lead to the development of calcium lactate crystals (Upreti and Metzger 2006).

Starter bacteria are integral to cheese manufacture and ripening. Their primary role is to acidify milk during the initial fermentation and then during ripening to lyse thereby releasing protease and lipase enzymes into the cheese matrix resulting in the breakdown of peptides, amino acids and fatty acids leading to the development of volatile flavour compounds (Hickey et al. 2015). The work of Jeanson et al. (2011) showed bacterial colonies to be randomly distributed and static within the cheese matrix, while other studies (Laloy et al. 1996; Lopez et al. 2006) showed bacteria to be located in direct contact with the MFGM or located at the casein-fat interface. During ripening, residual sugars such as lactose and galactose provide substrate for the growth of non-starter lactic acid bacteria (NSLAB), while

metabolites of starter fermentation such as lactate enable growth of secondary/adjunct cultures such as propionic acid bacteria.

Given the complexity of cheese as a physical and biological system and considering its manufacture from a raw material which can vary markedly in composition, there is a continued need to understand and control the factors that influence cheese quality and consistency. Traditionally, research in the area of cheese microstructure has relied on scanning or transmission electron microscopy and basic confocal microscopy of fluorescently labelled cheese samples. Scanning electron microscopy (SEM) provides detailed topographic images of the cheese surface (or fracture surfaces in the case of freeze-fracture cryo-SEM), differentiating fats, proteins salt crystals and even individual bacterial cells and colonies. However, standard electron microscopy cannot provide complete 3D information about spatial arrangement of individual cheese components. On the other hand, confocal laser scanning microscopy (CLSM), in conjunction with proper fluorescence staining, can provide three-dimensional visualization of fat and protein components or separation of dead and live bacteria inside the cheese matrix (Auty et al. 2001; El-Bakry and Sheehan 2014).

When detailed information about local chemical properties or spatial organization of individual cheese components is required, e.g. individual lipid constituents (triglycerides, phospholipids), distribution of calcium lactate crystals, local pH, or distribution of trace components, advanced microscopic techniques prove themselves to be valuable tools in food research. Thus, with recent development in materials sciences including advanced microscopic techniques, it is now timely to apply these techniques to gain a deeper understanding of cheese matrices and their role in cheese quality and consistency. The objective of this research was to evaluate the application of a range of advanced light microscopic techniques in research into the physicochemical properties of cheese matrices.

2 Materials and methods

2.1 Cheese preparation

2.1.1 Starter strains

Thermophilic starter cultures typically used in Swiss-type cheese manufacture (Scott 1981), i.e. *S. thermophilus* TH3 (ST) and *L. helveticus* LHB02 (LH), were purchased from Chr. Hansen Ltd. (Little Island, Co. Cork, Ireland) as individual frozen concentrates and stored at $-80\text{ }^{\circ}\text{C}$ until cheese manufacture.

2.1.2 Cheese manufacture

Raw milk was obtained from a local dairy company, standardized to a protein to fat ratio of 1.01:1, held overnight at $<10\text{ }^{\circ}\text{C}$, pasteurized at $72\text{ }^{\circ}\text{C}$ for 15 s, and pumped at $32\text{ }^{\circ}\text{C}$ into cylindrical jacketed stainless steel vat (500 L) with automated variable speed cutting and stirring equipment (APV, Schweiz AG, Worb, Switzerland). Cheesemilk was heated to $34\text{ }^{\circ}\text{C}$ and inoculated with $10\text{ mg}\cdot\text{L}^{-1}$ of frozen direct vat starter (DVS) ST and $5\text{ mg}\cdot\text{L}^{-1}$ of frozen DVS LH (Chr. Hansens Ltd.). After a 60-min ripening period, chymosin (Chymax plus, Chr. Hansens Ltd.) diluted to 1:6 with de-ionized water, was added at a level of 18 mL per 100 kg of milk. A coagulation time of 35 min was allowed prior to cutting of the

coagulum. After a 10-min heating period, the curd/whey mixture was cooked by steam injection into the jacket of the vat with constant stirring. Maximum scald employed was 50 °C. Curds were cooked at a rate of 0.5 °C.min from 34 to 45 °C and at 1 °C.min from 45 °C to maximum scald. Curds were pitched at pH 6.3.

The curds were drained, cheddared, milled at pH 5.3, salted at a rate of 1.45 % (w/w) and pressed overnight on a horizontal press at 264.6 kPa in 20 kg blocks. The cheeses were then vacuum packed and ripened at 8 °C for up to 230 days.

2.1.3 Cheese preparation for light microscopy

Thin slices of cheese, measuring approximately 5×5×3 mm, were freshly cut from a cheese sample using a scalpel.

Neutral lipids were stained with a lipid specific Nile blue fluorescent dye (Sigma-Aldrich, St. Louis, MO), prepared in acetone with concentration of 42 µg.mL⁻¹.

Phospholipids were labelled with N-(lyssamine Rhodamine-B sulfonyl)-1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (Rh-DOPE; Avanti Polar Lipids Inc., Birmingham, England), dispersed in chloroform. To stain phospholipids, about 200 µL of the staining of Rh-DOPE was added to the coverslip. After chloroform evaporation, the coverslip was pressed against the slice of cheese to permit diffusion of the stain for 30 min in the dark at 4 °C.

2.2 Advanced techniques in optical microscopy: basic principles and instrumentation

2.2.1 Confocal laser scanning fluorescence microscopy

Confocal laser scanning fluorescence microscopy (CLSM) has long been a standard tool in cell biology and became popular in other areas of science, including food research. It is based on fluorescence, a phenomenon where a photon of suitable wavelength is absorbed by a specific dye molecule, followed by an emission of a photon with somewhat longer wavelength (lower energy). With a set of fluorescence filters, the distribution of the fluorescent can be observed with high contrast and specificity. The main disadvantage of simple widefield fluorescence observation is the blur and haze caused by out-of-focus fluorescence, particularly in thick samples, a common situation in food research. The elaborate optical arrangement of a CLSM effectively blocks the out-of-focus light, employing laser excitation of a single spot in the sample, fast raster scanning in three dimensions and efficient multichannel detectors (Pawley 2006). With a CLSM, one obtains a full 3D representation of the spatial distribution of fluorescent molecules in the sample with high contrast and resolution.

The CLSM is a basic platform that can be easily extended to perform advanced microscopic imaging, such as two-photon excitation, second harmonic generation, fluorescence lifetime or Raman microscopy.

2.2.2 Fluorescence lifetime imaging microscopy

Fluorescence lifetime imaging microscopy (FLIM) measures the characteristic time a fluorescent molecule spends in the excited state (fluorescence lifetime). This lifetime is

typically a few nanoseconds in length and it is specific for given fluorophore. Moreover, the fluorescence lifetime of many fluorescent dyes is a sensitive indicator of local chemical properties, such as ion concentration, pH, oxygen concentration or presence of other fluorescent molecules (Lakowicz 2006). Consequently, FLIM can not only be used to distinguish different fluorophores on the basis of their characteristic lifetimes (rather than their spectral properties) but also to distinguish among different environments based on changes in lifetime of the same fluorophore, for example local pH within the cheese matrix (Burdikova et al. 2015). Fluorescence lifetime microscopy can also provide additional valuable information in fat composition studies when combined with various fluorescent dyes (e.g. Nile blue, Rhodamine-DOPE). Better understanding of the composition of lipid droplets in cheese matrix may improve the functional and nutritional properties of food products (Lopez and Briard-Bion 2007).

Fluorescence lifetime measurements of Nile blue- and Rhodamine-DOPE-stained cheese samples were performed with Zeiss LSM780 NLO confocal microscope based on Axio Observer. Z1 inverted microscope (Carl Zeiss MicroImaging, GmbH) equipped with Chameleon Vision (Coherent, Inc.) pulsed IR laser (0.1 ps pulses, 80 MHz repetition rate) tuned to 860 nm for two-photon fluorescence excitation and Becker & Hickl hybrid detector (HPM-100-40) and TCSPC hardware (SPC-150, Becker & Hickl, GmbH). Fluorescence was collected through an IR-blocking filter and 580–640 nm emission filter with a Plan Apo 63×/1.4 oil IR objective lens. The measured fluorescence decay curves were analyzed with Becker & Hickl SPCImage fitting software to yield the mean fluorescence lifetime in 256×256 pixel lifetime images.

2.2.3 Two-photon excitation and second harmonic generation imaging microscopy

Second harmonic generation (SHG) imaging is non-linear optical process with two requirements—high excitation intensity and non-centrosymmetric molecules with long range order present in the sample (Bianchini and Diaspro 2008). No fluorescence takes place in this process and the wavelength of the emitted light (as the name suggests) is exactly one half of the excitation wavelength. Generally, the same pulsed laser source used for two-photon excitation (TPE) is also suitable for SHG, and these two signals can be easily separated spectrally. As no labelling is needed for SHG imaging (Diaspro et al. 2002), it is a vital method for visualizing certain specific components of the sample, e.g. collagen in tissues (Filová et al. 2010; Šepitka et al. 2012). Some low symmetry crystals also emit strong SHG signal (Haupt et al. 2012) so this technique can be applied to detect calcium lactate crystals within cheese matrices.

TPE is a non-linear process where a fluorescent molecule simultaneously absorbs two photons of low energy (long wavelength, typically in the near infrared part of the spectrum) in order to reach an excited state. The dye molecule then emits a photon, whose energy is somewhat lower than the sum of the excitation photon energies (the emission wavelength is longer than one half of the excitation wavelength). However, for this phenomenon to appear, the excitation intensity must be exceptionally high (megawatts per square micrometre) and this is only achievable with dedicated pulsed lasers (pulses of the order of picoseconds or shorter) in a laser point scanning setup (Diaspro et al. 2006). The main advantages of two-photon excitation microscopy are (i) lower absorption and scattering of the IR excitation light in many kinds of samples (e.g.

biological tissues), (ii) due to non-linear effects, no fluorescence originates from outside the focus plane (Burdiková et al. 2010), so there is no need to block the out-of-focus light, and this greatly enhances the fluorescence collection efficiency in highly scattering samples. These two effects together facilitate fluorescence microscopy deep within a scattering sample.

SHG signal of calcium lactate crystals, together with TPE fluorescence, were collected with TCS SP8 AOBS MP confocal microscope (Leica Microsystems, GmbH) equipped with InSight DeepSee (Spectra-Physics, Inc.) pulsed IR laser (0.1 ps pulses, 80 MHz repetition rate) tuned to 860 nm. Internal spectral detectors were used for fluorescence acquisition and the frequency doubled signal was observed through 420–440 nm bandpass filter in transmission geometry.

2.2.4 Raman confocal imaging

Raman scattering is a process of inelastic scattering of photons, where part of the photon energy is transferred to molecular vibrations. It is a very weak process (typically less than 1 ppm of incoming photons are inelastically scattered), however, the spectrum of the scattered photons (Raman spectrum) shows a very high level of detail. Every molecule in the sample has its characteristic spectrum, reflecting the energies of vibrational modes of that molecule. With known spectra of individual constituents (often found in specialized databases), one can decompose the measured Raman spectrum (using, e.g. principal component analysis (PCA) (Shinzawa et al. 2009)) into the contributions of the individual chemical species. When combined with a confocal microscopic setup, the method (Hollricher and Ibach 2011) is a powerful tool to determine the chemical composition of the sample with micrometre resolution, in 3D and without any labelling. Here, we show the potential and limitations of this technique in determining the local composition of cheese samples.

A slice of cheese was cut using a sharp knife and the local Raman spectrum was taken using inVia confocal Raman microscope (Renishaw, PLC) with 785 nm excitation (laser power of about 60 mW in the sample plane) and 20 \times objective. Laser spot is visible in the middle of Fig. 5. Vertical size of the laser spot is about 20 μ m.

In order to extract quantitative information from the acquired Raman spectra, the statistical classification of the spectra was carried out adopting a rolling-circle procedure for spectral processing. In this way, the slowly varying spectral background is suppressed. Also automated subtraction of cosmic ray peaks, and the Savitzky–Golay procedure for subsequent noise filtering was employed. Finally, all the data obtained from the studied samples was analyzed using standard principal component analysis (PCA) algorithms in order to distinguish possible difference between them.

2.2.5 Cryo-electron microscopy

Cheese samples were imaged in a scanning electron microscope using an Alto2500 cryo-stage system (Gatan UK Ltd., Abingdon, UK.). Sections of cheese 1 \times 1 \times 4 mm were cut at room temperature with a razor blade, then plunge frozen in liquid nitrogen slush at -207 $^{\circ}$ C, followed by freeze fracturing under vacuum in the cryo-chamber using a precooled scalpel blade. The fractured surfaces were then etched at -85 $^{\circ}$ C for 15 min, cooled to -125 $^{\circ}$ C, and coated with sputtered platinum (10 mA for 60 s).

Samples were transferred under vacuum to the cold stage in a Zeiss Supra 40VP field emission scanning electron microscope (Carl Zeiss AG, Darmstadt, Germany), where they were maintained at $-125\text{ }^{\circ}\text{C}$ and imaged using an accelerating voltage of 1 kV.

3 Results and discussion

3.1 Calcium lactate crystals visualized with SHG microscopy

To our knowledge, it is the first time the SHG microscopy has been applied to examine dairy matrices of any product type and in particular to visualize calcium lactate crystals placed inside cheese matrix without labelling. In Fig. 1, we compare SHG images of the crystals, overlaid with TPE fluorescence of the cheese matrix (Fig. 1a, see also the animation in Online Resource 1) with the already established methodology in cheese research—cryo-SEM (Fig. 1b). The lower resolution of SHG is clearly offset by straightforward sample preparation, the depth resolution and the possibility to combine SHG with other fluorescence staining methods (e.g. Nile blue) and to study the mutual spatial organization of the individual cheese components.

The SHG signal also depends on the polarization of excitation light with respect to crystallographic orientation of the crystals. To demonstrate this, we rotated the polarization of the excitation light and acquired the SHG images at angles 0° (green), 30° (red) and 60° (blue), Fig. 2a, b. The merge of all three angles provides complex information about the lactate calcium crystal location, shape and orientation with particular reference to the protein and fat components of the cheese matrix. Thus, it provides a greater visual understanding of the relationship between calcium lactate crystals and other constituents of the cheese matrix.

Calcium lactate crystals form when Ca^{2+} and lactate ions exceed their solubility, supersaturate the serum phase of the cheese and then crystallize at nucleation sites within cheese matrices. Most starter bacteria ferment lactose to L-lactate, however, considerable amounts of D-lactate are formed by the activity of NSLAB during cheese ripening. The lower solubility of Ca-D-lactate then causes crystal formation (Lawrence et al. 2004). Crystallization of calcium lactate on the surface of Cheddar cheese is a

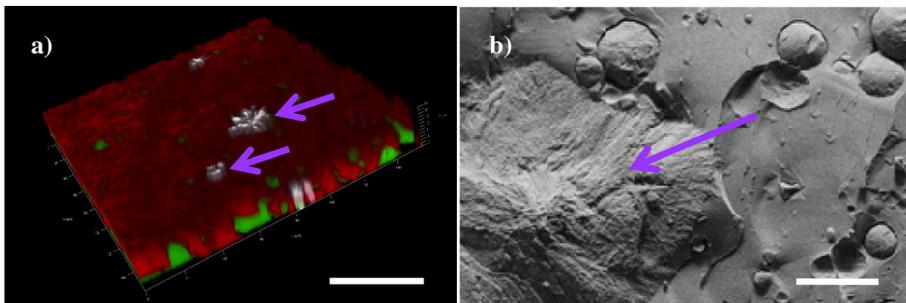


Fig. 1 Calcium lactate crystals inside the Cheddar cheese matrix. **a** (red) two-photon fluorescence of protein matrix stained with Nile blue; (green) two-photon fluorescence of fat stained with Nile blue; (grey) SHG signal from the calcium lactate crystals (purple arrows); scale bar 50 μm . **b** Freeze-fracture cryo-SEM image showing the details of fat droplets in protein matrix and a single calcium lactate crystal (purple arrow); scale bar 5 μm

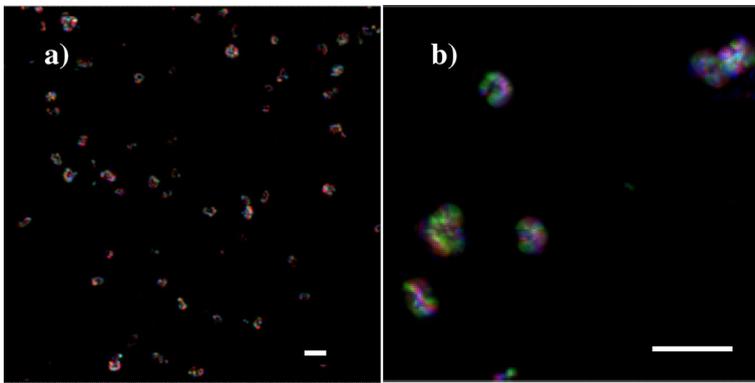


Fig. 2 **a** Calcium lactate crystals inside the Cheddar cheese matrix visualized using SHG microscopy technique. The polarization angles of the incident light were color coded as *green* (0°), *red* (30°) and *blue* (60°). **b** Detail of individual crystals. *Scale bars* 20 μm

common and troublesome defect (Pearce et al. 1973). The deposits are visible to the naked eye; products containing crystal deposits are not aesthetically pleasing and can be sometimes rejected by the retailer/consumer who may confuse them with mould contamination.

Calcium lactate crystals can be visualized using X-ray crystallography but we will miss the chemical information about the placement fat and protein around the crystals. The crystalline deposits are also frequently observed by standard light microscopy SEM or TEM in processed cheese products (Kalab 1995). Using various techniques such as electron microscopy and X-Ray diffraction analysis, infrared spectroscopy and energy dispersive X-Ray spectrometry analysis (EDSA), several crystal types have been identified, e.g. monoclinic calcium pyrophosphate dehydrate, disodium phosphate dodecahydrate, unreacted melting salts, tyrosine, calcium citrate, fatty acids, protein and lactose (Kondo et al. 1990). The composition of crystalline structures in natural cheeses and PCPs was studied by with SEM EDSA and X-Ray diffraction (Washam et al. 1985). Calcium specific fluorescent stain Alizarin Red was used to identify calcium lactate crystals by optical microscopy (Yiu 1985).

In comparison, SHG microscopy does not allow us to chemically characterize the composition of crystals, but this label-free microscopic technique allows us to visualize the calcium lactate crystals inside cheese matrix, and with combination with standard CLSM, we can get also the information about the relationship between crystals and the surrounding environment like location of fat, protein, phospholipids, live/dead bacteria in three dimensions (Online Resource 1). This localization after further investigation can further illustrate the relationship between calcium lactate crystals and bacterial, fat and mineral placement inside the cheese matrix.

3.2 Lipids visualized with FLIM

It is strongly evident that the sensitivity of the spectral properties of Nile blue fluorescent dye towards the polarity of the local environment (Jose and Burgess 2006), as shown in Fig. 3a, b, enables the analyst to determine with much greater clarity and precision the dispersion and morphology of fat within the cheese matrix. As

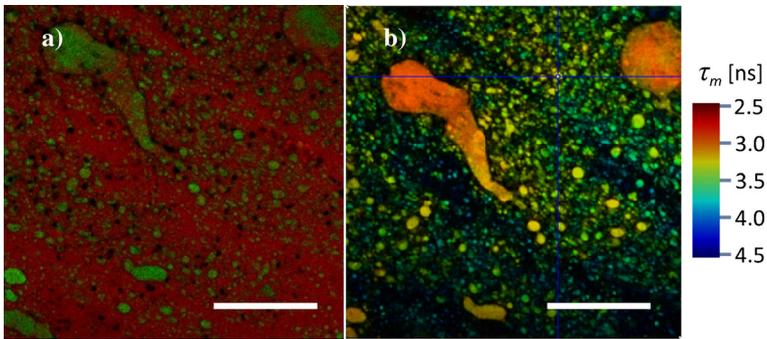


Fig. 3 **a** Confocal image of the Cheddar cheese surface; Nile blue staining: (green) fat, (red) protein matrix. **b** Fluorescence lifetime of the Nile blue-stained cheese surface in the 520–570-nm emission band, corresponding to non-polar (fat) environment. The pseudocolor encodes the local fluorescence lifetime. Scale bars 20 μm

expected, fat in the cheese matrix illustrated in Fig. 3 is comprised of both fat in globular form and non-globular free fat. It may also be considered that the results of our FLIM studies of the lipid phase show strong correlation between the fluorescence lifetime and the size of the individual lipid droplets (Fig. 3b). This phenomenon deserves more investigation, as it may suggest that the composition of lipid droplets may depend on their size, or vice versa.

Based on the work of Lopez et al. (2008), we also applied the fluorescent marker Rhodamine-DOPE for phospholipids, i.e. lipids that can form lipid bilayers due to the presence of hydrophilic head, in addition to their hydrophobic tail. Figure 4a, b acquired by CLSM show the occurrence of phospholipids on the edge between the fat droplets and the protein matrix. The corresponding FLIM image shows additional details of phospholipids placement (Fig. 4c), additional visual information is conveyed by the pseudocolor, encoding the local fluorescence lifetime.

Currently it is possible to detect phospholipids using spectroscopy, nuclear magnetic resonance spectroscopy, particularly with P-NMR. With HPLC-ELSD, it is possible to determine the composition and type of phospholipids present, but it does not provide information about the placement and location of the phospholipids in within the cheese matrix. Utilization of FLIM methodology provides more accurate information about the placement of phospholipids in the cheese matrix. To achieve separation of different types of fats inside the cheese matrix, we used the same methods as used previously (Lopez et al. 2008) for CLSM.

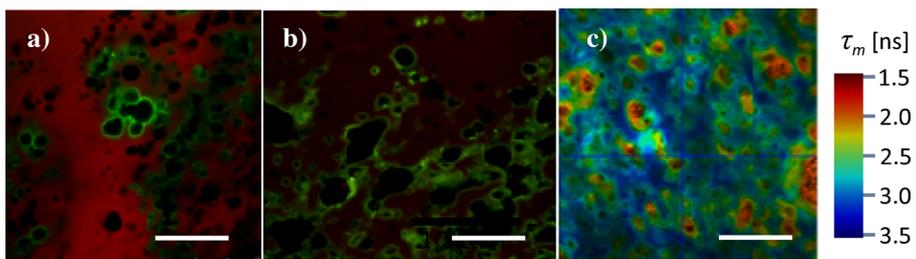


Fig. 4 **a, b** Phospholipids visualized by Rhodamine-DOPE (green) and protein matrix (red), Cheddar cheese, CLSM; **c** FLIM image of the phospholipids (Rhodamine-DOPE). The pseudocolor encodes the local fluorescence lifetime. Scale bars 20 μm

3.3 Characterization of chemical composition of cheese matrix by confocal Raman microscopy

This method offers the capability to determine variability in chemical compositions arising during cheese manufacture, which in the long term, offers the potential for developing rapid or real-time in situ process analysis and control methodologies. Moreover, we have demonstrated that it is possible to reveal the contribution of fatty acids and proteins (Figs. 5 and 6) to the overall statistical variance within the chemical fingerprint using PCA so that different types of cheese can be distinguished. The results suggest that Raman spectroscopy analysis has the potential for the characterization of cheese without any time-consuming sample preparation (see sample preparation for experiments at Fig. 5). This could be of benefit in the food industry where Raman spectroscopy can be used as a quality sensor of selected products directly at the production line rapidly sensing any changes in chemical composition.

To demonstrate the capability of Raman spectroscopy for the compositional analysis and the spatial visualization of cheese samples, we have recently performed experiments in which spectra were acquired point-by-point, at a few selected positions on a cut sample surface (Fig. 5). Consequently, some relevant molecular complexes (Fig. 6) could be identified in this way, with some spatial resolution so that e.g. inhomogeneous distribution of beta-carotenoids is visible. As an example, this analytical approach could help to determine the underlying causes of the development of a pink color defect, persistent in a wide range of ripened cheese varieties (Daly et al. 2012).

Raman spectroscopy allows the clear identification of particular biological molecular compounds within a complex spectrum which are responsible for changes in the

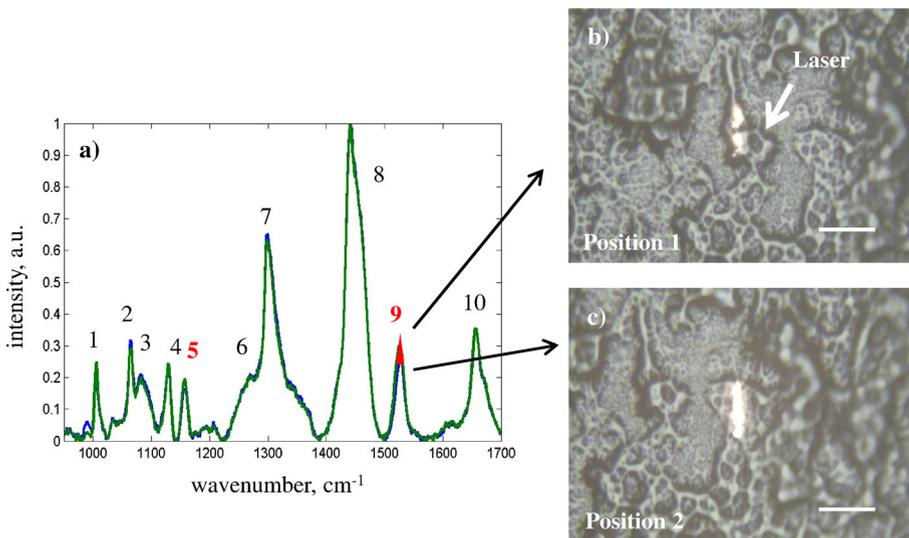


Fig. 5 a Raman spectra of Cheddar cheese taken from two different positions of a cut cheese surface; b, c positions from which the spectra were taken—in the middle of each position, a laser trace is shown (this is the exact location from which data were taken). Note the *arrows* showing differences in beta-carotene peaks—indicating its inhomogeneous distribution. The vertical size of the laser spot is about 20 μm . Samples for analysis were achieved by preparing very thin slices and placing directly under a microscope where Raman spectrum was taken. *Scale bars* 20 μm

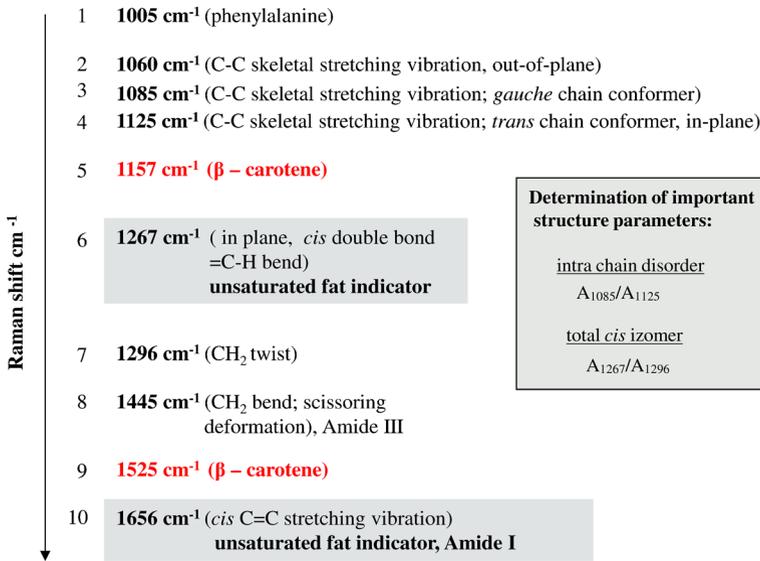


Fig. 6 Summary of prominent peaks observed in the Raman spectra of Cheddar cheese, together with suggested assignment of chemical compounds. The *peak numbers* of the table are used to identify features in the spectra shown in Fig. 5. Determination of selected structure parameters is highlighted in the *inset*

matrix (for example, in cheese matrices fats and protein). Raman spectroscopy can be regarded as the method of choice for analysis of biological/food samples (Maquelin et al. 2002; Harz et al. 2005; Bernatová et al. 2013; Samek et al. 2014). Two previous reviews provide valuable information on Raman spectroscopy studies of the molecular complexes in biological specimen (Movasaghi et al. 2007; Notinger 2007). In

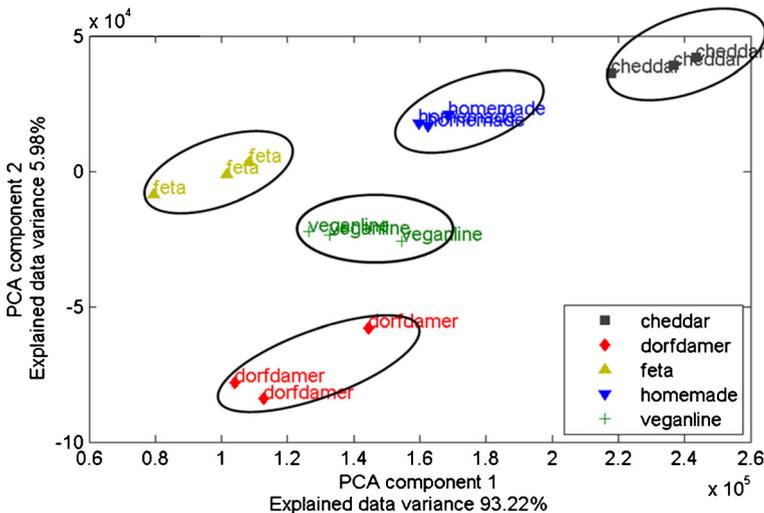


Fig. 7 Scores plot of the first two principal components for five different types of cheese. With PCA, one can clearly separate the clusters of spectra associated with the different types of cheese. Cheese types were selected/bought in the local shop as follows: Cheddar, home-made cheese from a local farmer, Dorddamer, Feta and Veganline

addition, a reasonably detailed database of Raman features encountered in biological samples was published (De Gelder et al. 2007). Subsequent spectral decomposition or blind principal component analysis (PCA) can stand as a basis for discerning important features of the studied samples, as exemplified in Fig. 7.

However, this type of localized investigation for species and/or compound identification is time consuming and often does not collect sufficient or complete (multi-spectra) information about the whole sample, particularly where there is likely to be inhomogeneity within the sample. A possible solution to the problem of localized measurements, to build up a compositional map, has come about with the commercial introduction of line-scan techniques. This approach is around for some time, and has recently been applied to the study of biological samples (Samek et al. 2010). In essence, rather than measuring the sample point-by-point the technique relies on line scanning in which multiple locations are sampled simultaneously. With the many, rapidly acquired spectra—up to tens of thousands of spectra—‘data mining’ for analysis and identification becomes possible, using standard chemometric techniques. This can be also combined with confocal imaging to quickly build up 3D maps of the surface.

4 Conclusion

Given the desire within the cheese industry to achieve a greater understanding of the influence of manufacture and ripening parameters on cheese quality and consistency, there is a continued interest in exploiting new analytical techniques to gain new insights into the physicochemical characteristics of cheese matrices. In this study, a range of advanced light microscopic techniques were evaluated to determine their potential to provide a better understanding of cheese matrices at the micro-scale and in particular to aid research into determining the level of localized inhomogeneity within the cheese matrix. This study concluded that (i) second harmonic generation microscopy technique facilitates visualization of calcium lactate crystals with reference to the fat and protein components within the cheese matrix; (ii) two-photon excitation fluorescent lifetime microscopic techniques enables a more precise determination and location of fats and phospholipids within the cheese matrix, and (iii) the Raman confocal microscopy offers a new and very powerful methodology to analyze and fingerprint different chemical components of the cheese matrix.

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Conflict of interest Zuzana Burdikova, Zdenek Svindrych, Cian Hickey, Martin G. Wilkinson, Mark A. E. Auty, Ota Samek, Silvia Bematova, Vladislav Krzyzaneck, Ammasi Periasamy, and Jeremiah J. Sheehan declare that they have no conflict of interest.

Statement of human and animal rights This article does not contain any studies with human or animal subjects performed by any of the authors.

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