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Comparison of the carotenoid profiles of commonly consumed smear-ripened cheeses

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Abstract

The objective of this study was to identify the carotenoids imparting the orange colour to the rind, and pale yellow color to the core, of selected smear-ripened cheeses. The cheeses investigated were Charloe, Ashbrook, Taleggio, and Limburger, and were sourced from artisanal markets. Samples of the rind and core were extracted using non-polar solvents, followed by saponification to hydrolyze triglycerides to remove fatty acids, and to release carotenoid esters. Extracts were tested using ultra-high pressure liquid chromatograph-diode array detector-high resolution mass spectrometry (UHPLC-DAD-MS and -MS/MS), and identities of α- and β-carotene, lycopene, and β-cryptoxanthin confirmed with authentic standards. β-Carotene was the predominant species in both the rind and core, absorbing ~70% of the signal at 450 nm in all cheese extracts tested, as well as minor quantities of β-cryptoxanthin and α-carotene. Carotenoids unique to the rind included lycopene as well as the rare bacterial carotenoids previously identified in bacterial isolates of cheeses (i.e. decaprenoxanthin, sarcinaxanthin, and echinenone). This is the first detailed characterisation of carotenoids extracted directly from smear-ripened cheeses, and reveals that smear-ripened cheese can contribute both provitamin A carotenoids as well as C50 carotenoids to the human diet.

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The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Keywords

Ashbrook; Charloe; Taleggio; Limburger; high performance liquid chromatography-mass spectrometry

1. Introduction

The diversity of cheese available in the market is influenced by various factors such as type of milk, season and process of manufacture, starter and adjunct cultures, ripening conditions, etc. (Almena-Aliste & Mietton, 2014). Biochemical factors like glycolysis, lypolysis, proteolysis, loosing or retaining of moisture, pH, oxygen permeability etc. highly contribute to flavor development (Manzo et al., 2019). Cheese can be classified based on the type of coagulation i.e., acid, rennet and a combination of heat and acid. Smear-ripened cheeses are rennet-coagulated cheeses with orange-red-brown colour on the surface, which is a characteristic feature of this variety of cheeses. Examples of smear-ripened cheeses include Limburger, Tilsit, Taleggio, Livarot, and Brick. Young smear-ripened cheeses are often inoculated with old brine, known as 'old-young' smearing. This process transfers microbes present in the brine solution to the surface of young cheeses (Cogan, 2014). Intermittent washing of the cheese surface with the brine solution results in an even distribution and development of the smear. Cultures deliberately added or originating as adventitious microbes during manufacturing and ripening, such as *Debaryomyces hansenii*, *Brevibacterium linens* and *Geotrichum candidum*, may also contribute colour to the finished product of some smear-ripened cheese like Limburger and Taleggio (Giuffrida et al., 2020).

The development of a smear on the cheese is reflected by an initial dominance of yeasts, including *D. hansenii*, *Saccharomyces*, *Candida*, *and Kluyveromyces*, to a level of~10^8cfu/g of cheese within 7 days of manufacture. These yeasts are responsible for the initial metabolic activities observed, driving functions such as the de-acidification of the cheese surface, whereby pH increases due to metabolism of lactate to CO_2 and H_2O (Mounier et al., 2006). These yeasts also induce the synthesis of some growth factors, like pantothenic acid, which promote the growth of less acid-tolerant but more proteolytic and salt tolerant Gram-positive bacteria, e.g., *Micrococcus*, *Corynebacterium*, *Glutamicibacter*, *Staphylococcus*, *Brevibacterium* and others (Bockelmann, 2002). Some non-salt tolerant yeasts may lyse during the early stages of ripening, releasing peptides, nucleotides, and vitamins (Sheehan 2007). Sources for these bacteria found in cheese include milk, brine, vats, ripening rooms and wooden shelves. The interactions between the aforementioned surface microbiota significantly contribute to flavor development through the diffusion of the products of enzymatic activities into the cheese core. These interactions are also important for developing the characteristic coloured surface of smear-ripened cheeses, where this colour is due to carotenoids produced by surface microbes (Corsetti, Rossi, & Gobbetti, 2001).

There are more than 1190 naturally occurring carotenoids originating from plants, algae and bacteria (Yabuzaki, 2017). Because animals are incapable of synthesizing carotenoids, they source carotenoids through their diet. The majority of carotenoids found in foods are C_{40} tetraterpenoids, with an extensive conjugated double bond system reflecting the yellow,

orange and red colours we associate with these compounds (Rodriguez-Amaya & Kimura, 2004). There is a wide range of health benefits associated with carotenoids. A subset of the "carotenes" class (i.e. those carotenoids composed strictly of hydrogen and carbon) with at least one unsubstituted β-ionone ring act as precursors for vitamin A. Vitamin A is an essential nutrient that plays a key role in cell differentiation, optimal functioning of the immune system, vision, and in adipose tissue regulation (Blaner, 2019; Britton, 1993; Harrison & Kopec, 2018). Other non-provitamin A carotenoids have been associated with health benefits as well, including the reduced risk of macular degeneration and improved cognitive function that are associated with the carotenoid lutein (Johnson et al., 2008; Sabour-Pickett, Nolan, Loughman, & Beatty, 2012), and the protection against certain types of cancer and cardiovascular disease that is associated with the carotenoid lycopene (Giovannucci, 2005; Sesso, Buring, Norkus, & Gaziano, 2004; Story, Kopec, Schwartz, & Harris, 2010). Carotenoids also have a variety of industrial applications as food colorants, nutrient supplements, animal feed, and in cosmetics and pharmaceutical products (Mathews-Roth, 1993; Mortensen, 2006; Nolan et al., 2016; Pickworth, Loerch, Kopec, Schwartz, & Fluharty, 2012). Smear-ripened cheeses may contribute to the dietary intake of common carotenoids in humans, and may also be a unique source for novel carotenoids specific to yeasts and bacteria particular to this family of cheeses. To further investigate these possibilities there is merit in identifying the carotenoids found on the surface of smear-ripened cheeses.

Various studies have endeavored to determine the type of carotenoids produced by the biomass of specific microbial isolates from the smear of smear-ripened cheeses (Giuffrida et al., 2015; Guyomarc'h, Binet, & Dufosse, 2000; Krubasik et al., 2001; Netzer et al., 2010; Tao, Yao, & Cheng, 2007). Formerly, *Brevibacterium* was regarded as being a major contributor, due to an ability to produce orange coloured C_{40} carotenoids like isorenieratene, 3'-hydroxy-isorenieratene, and 3'3'-dihydroxy-isorenieratene (Guyomarc'h et al., 2000; Kohl, Achenbach, & Reichenbach, 1983). Later it was established that the contribution of *B. linens* to the orange colour of smear cheeses could only be possible when the species is present at 5% of the total microbial population (Bockelmann, 2002), which rarely occurs. Recent advances in molecular methods such as cloning and sequencing were used to characterize the diverse microbial biodiversity of the smear surface, and C_{50} carotenoid production was identified in bacterial species related to smear bacteria. These C_{50} carotenoid-producing species, which occupy the majority of the cheese surface by the end of ripening, are *Micrococcus*, *Glutamicibacter*, *Dietzia*, and *Corynebacterium* (Giuffrida et al., 2015; Heider et al., 2012; Netzer et al., 2010; Tao et al., 2007). Interactions between these bacteria and other bacteria, yeasts, and casein hydrolysates from cheese (Bockelmann, 2002), under different pH, NaCl concentrations and temperature (Masoud & Jakobsen, 2003, 2005), can also influence colour development.

Despite this indirect evidence, and despite the existence of a number of strategies to extract carotenoids from the cheese matrix (Higuchi & Peterson, 1946; Hulshof, van Roekel-Jansen, van de Bovenkamp, & West, 2006; Lucas, Coulon, Agabriel, Chilliard, & Rock, 2008; Ollilainen, Heinonen, Linkola, Varo, & Koivistoinen, 1988), few reports have successfully directly identified the carotenoids present within smear-ripened cheeses (Galaup et al., 2007, 2015). Thus, the aim of the current research was to identify and compare the carotenoid

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profiles of a variety of commercially available smear-ripened cheeses. To facilitate this, a hybrid combination of the previously published non-polar liquid-liquid extraction methods was used to remove carotenoids from the cheese rind and core, and extracts were analyzed via ultra-high performance liquid chromatography-diode array detection-high resolution mass spectrometry (UHPLC-DADMS and UHPLC-DAD-MS/MS).

2. Materials and Methods

2.1. Chemicals

Optima grade formic acid and β-carotene standard (97% purity) were purchased from Sigma-Aldrich (St. Louis, MO). A lycopene standard was isolated and purified following a previously published method (Kopec et al., 2010). β-Cryptoxanthin (97% purity) was purchased from Extrasynthese (Genay, France). Lutein (95% purity), zeaxanthin (98% purity), and α-carotene (95% purity) were purchased from Cayman Chemical (Ann Arbor, MI). Optima grade methanol, HPLC grade acetone, hexane, and methyl *tert*-butyl ether (MTBE), and ACS grade potassium hydroxide were purchased from Fisher Scientific (Pittsburgh, PA). Double-deionized water was obtained from a Millipore Q-Plus filtration system.

2.2. Samples

Smear-ripened cheeses (Figure 1) were purchased at local artisanal markets. Cheeses were chosen on the basis of their origin and type of milk used. Ashbrook (from a producer in Reading, VT) and Charloe (from a producer in Defiance, OH) are made from raw cow's milk. Taleggio, a protected designation of origin cheese (from a producer in Lombardy, Italy), and Limburger, a style of cheese originating from Belgium (from a producer in Monroe, WI), were made from pasteurized cow's milk.

2.3. Extraction of carotenoids from the cheese matrix

A figure of the experimental design is shown in Figure 2. Rind (2 g) was carefully scraped from the cheese exterior with a depth of ~2 mm, or a core sample (2 g) taken from the interior, were blended with 10 mL of methanol using a homogenizer (PowerGen 500, Fisher Scientific, San Diego, CA), in a 50 mL falcon tube. Samples were probe sonicated for 30 s on ice, followed by centrifugation at 2147 × *g* (3000 rpm) for 10 min. The supernatant was decanted into a separate 50 mL glass vial, and to the remaining cheese pellet, 10 mL of acetone/hexane (1:1) was added. This mixture was again sonicated and centrifuged as described, with the supernatant decanted into the 50 mL glass vial containing the methanol. This step was repeated until no color remained in the cheese pellet (approximately 5 times). One mL of saturated aq. NaCl solution was added to the pooled supernatant, followed by mixing and centrifugation for 5 min, to induce phase separation. Hexane aliquots (2 mL) were placed in glass vials and saponified by adding 2 mL of a 20% methanolic KOH solution and stirred vigorously for 2 h at 4 °C. After saponification, the hexane layer was dried under argon gas and stored at −20 °C for not more than 24 h before analysis. Samples were reconstituted in 1:1 MTBE/methanol and vortexed for 30 s before UHPLC-DAD-MS analysis.

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2.4. UHPLC-DAD-MS and MS/MS analyses

Separation of the cheese extracts was performed on an Agilent UHPLC 1290, either with an Agilent Eclipse XDB-C18 column, 150mm × 4.6 mm i.d., 5 μm particle size, or with a YMC C30 column, 150 mm × 4.6 mm i.d., 3 μm particle size. The composition of solvent A was 80:20 methanol/water and B was 78:20:2 MTBE/methanol/water, with 0.1% (*v/v*) formic acid added to both solvents as a modifier. The gradient was as follows: beginning at 5% B at 0 min, with a linear increase to 95% B over 18 min, holding for 3 min at 95% B, and immediately returning and holding at 5% B over 3 min. A flow rate of 1 mL/min with a column temperature of 40 °C was used. The UHPLC was interfaced with a QTof 6545 (Agilent Technologies, Santa Clara, CA) using an atmospheric pressure chemical ionization (APCI) probe operated in both positive and negative modes. Additional source settings were as follows: source gas = N_2, gas temperature = 350 °C, vaporizer temperature = 350 °C, desolvation gas flow = 8 L/min, nebulizer gas = 241 kPA. The mass spectra were acquired with a scan range of *m/z* from 100 to 700. Authentic standards of α-carotene, β-carotene, lycopene, β-cryptoxanthin, lutein, and zeaxanthin were used to identify these carotenoids in the samples. A tangerine tomato extract rich in phytoene, phytofluene, and neurosporene was used as a pseudo-standard to determine if these carotenoids were present in the cheeses. The remaining carotenoids were tentatively identified based upon anticipated retention times relative to the known carotenoids in the sample, as well as UV-Vis spectra, MS precursors, and MS/MS product ions coinciding with those reported in the literature.

3. Results

3.1. Carotenoids identified in rind extract

HPLC-DAD chromatograms of the rind extracts of all four cheeses at 450 nm as separated on the C18 column are shown in Figure 3. The same extracts were also separated on the C30 column, as shown in supplementary Figure S1. To further characterize the cheese rind carotenoids, MS/MS fragmentation was performed using APCI ionization in both positive and negative modes. The UV-Visible spectra, retention order, the primary precursor ion (*m/z*), and product fragments (*m/z*) are shown in Table 1.

The rind carotenoid profiles of all cheeses were very similar, with ~17 compounds eluting between 11.7–17.7 min. Regardless of cheese type, ~80% of all peak area at 450 nm was determined to be a mix of α- and β-carotene (see Figure 3), as confirmed with authentic standards using the C18 method. Further analysis with the C30 column revealed that 15% of the total carotene peak was α-carotene, with the remaining 85% as β-carotene (Figure S1). Both *trans*- and c*is*-lycopene, co-eluting with a flavuxanthin (an intermediate in the biosynthesis pathway of decaprenoxanthin/sarcinoxanthin (Heider et al., 2012; Netzer et al., 2010), were also observed in the rind samples. Likewise, the C50 carotenoids decaprenoxanthin and sarcinaxanthin (both free or esterified) were observed in the rind (but not core) extracts all cheeses tested. Decaprenoxanthin comprised ~0.8% and sarcinaxanthin ~1.45% of the entire signal at 450 nm. Although they share the same precursor *m/z*, the relative differences in UV-visible spectra (i.e., 2 nm hypochromic shift, Figure 4) and retention order, provided the tentative assignments (Enzell & Francis, 1969; Giuffrida et al., 2015; Norgard, Francis, Jensen, & Liaaen-Jensen, 1970). Fragment ions for

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decaprenoxanthin were *m/z* 687.5 $[M+H-H_2O]^+$ representing the loss of water, *m/z* 613.5 $[M+H-92]^+$ representing the loss of toluene, *m/z* 595.4 $[M+H-18–92]^+$, *m/z* 547.5 $[M+H-158]^+$ corresponding to the loss of dimethylcylodecapentaene (Arpin, Liaaen-Jensen, & Trouilloud, 1972; Enzell & Francis, 1969), and *m/z* 669.6 $[M+H-35]^+$, a fragment also reported by Giuffrida et al., 2015, but whose identity has not been determined.

With regards to sarcinaxanthin, the precursor consisting of a water loss was more intense than the protonated species. Fragments ions *m/z* 595.4 $[M+H-18–92]^+$ and *m/z* 473.1 $[M+H-92–140]^+$ are consistent with a toluene loss (Liaaen-Jensen & Hertzberg, 1968). Similarly, analytes with UV-Vis spectra and precursor *m/z* of 705.5 $[M+H]^+$ were observed eluting later in the method (see Table 1, peaks 8, 16, 17) suggesting mono- and di- esterified decaprenoxanthin and sarcinoxanthin were present in the rind sample, but the precursor ion signal in negative mode was not strong enough to identify the fatty acid moiety attached.

3.2 Carotenoids identifed in core extracts

HPLC-DAD chromatogram profiles obtained from the C18 separation of the 2 g of core extracts are presented in Figure 5, utilizing the same procedure as rind sample detailed above. Provitamin A carotenes (both α- and β-carotene listed as peak 15 in Table 1) were the dominant carotenoid class observed in core samples, absorbing ~98% of all signal at 450 nm. Minor compounds included β-cryptoxanthin, *cis*-β-carotene and β-cryptoxanthin, and tentatively epoxy-β-cryptoxanthin, an unknown, echinenone, epoxy-β-carotene, at negligible levels.

3.3. Minor carotenoids identified in rind and core extracts

Echinenone was observed in all four cheeses, with the UV-Vis spectrum, precursor, and productions consistent with literature values (van Breemen, Dong, & Pjkovic, 2012). Likewise, UV-Vis, precursor and product ions were consistent with epoxy-β-carotene in all four cheeses (De Rosso & Mercadante, 2007), with fragment *m/z* 461.3 $[M+H-92]^+$ revealing a toluene loss and fragment of *m/z* 205.1 corresponding to the epoxy group at the end of a polyene chain, and fragment *m/z* 177.1 corresponding to 9–10 bond cleavage (Enzell & Francis, 1969; van Breemen et al., 2012).

Esters of α-and β-cryptoxanthin were identified via UV-Vis spectra, elution times relative to one another (i.e. α- eluting before β-cryptoxanthin ester (peak 11)), and precursor ions matching β-cryptoxanthin standard (precursor signal was too low for fragmentation).

Other compounds remain unknown. Peak 3' was observed in Limburger and at much lower concentrations in Charloe. Peak 3 at 12.74 min was observed in Ashbrook, Limburger, and Taleggio, and whose UV-Vis spectral fine features included a rounded peak at a $\lambda_{max} = 458$ nm.

4. Discussion

A detailed investigation of the carotenoids present in smear-ripened cheeses was performed. Carotenoids identified in both rind and core samples included the pro-vitamin A carotenoids, most dominantly β-carotene, as well as α-carotene, β- and α-cryptoxanthin. Previous studies

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suggests that the influence of the seasonal milk, feed provided to cows, manufacturing and ripening processes, pasteurization, and standardization of milk will influence the levels of carotenes (e.g. β-carotene, α-carotene) xanthophyll (e.g. lutein, zeaxanthin, α- and β-cryptoxanthin) in milk and in milk products (Higuchi, Price, & Peterson, 1946; Higuchi & Peterson, 1946; Hulshof et al., 2006; Lucas et al., 2008). In contrast, certain carotenoids were only observed in the rind samples, including decaprenoxanthin, sarcinaxanthin, and their esters, as well as *cis*- and *trans*- lycopene. Both lycopene and the C50 carotenoids identified in the rind reflect a red hue, in contrast to the orange reflected by provitamin A carotenoids found both in the core and the rind, and are likely partially responsible for the darker color of the rind.

With the vast difference in milk sources, location of cheese producers, and differences in cheese styles articulated in the introduction, it was surprising that there was such limited variation between the cheese rind and core profiles, respectively, observed in this analysis. However, this result is consistent with a previous work which identified only seven distinct carotenoid profiles of from 114 bacterial strains individually isolated from a selection of French smear-ripened cheeses (Galaup et al., 2005).

Carotenoid extraction from the cheese matrix proved to be challenging. The very large quantity of lipid in the extract did not permit analysis without saponification, and utilizing this strategy increased sample concentration by ~10 fold, permitting carotenoids to be observed in the chromatograms. Saponification protocols of (Ollilainen et al., 1988) and (Jacobo-Velázquez & Hernández-Brenes, 2012) were tested, but these methods did not improve our sensitivity. Ultimately, the lipid remaining in the saponified extract produced large quantities of matrix interference which hampered MS ionization. This issue, combined with a low abundance in our samples, caused some carotenoids to remain only tentatively identified or un-identified. It should also be noted that even with temperature control, the saponification and sequential concentration steps may have also produced the epoxides and *cis*-carotenoids observed. Likewise, although strong saponification conditions were used, multiple esters were observed, suggesting saponification was only partially completed.

C_{50} bacterial carotenoids (i.e., decaprenoxanthin, sarcinaxanthin) were tentatively identified in the rind, as well as their presumed esters. Decaprenoxanthin was initially isolated from *Agromyces mediolanus* (formerly known as *Flavobacterium dehydrogenes*) (Goldstein, Halpern, & Robert, 1967). Later a detailed biosynthetic pathway was identified in *Corynebacterium glutamicum* (Krubasik et al., 2001). Three-pronged UV-Visible spectra consistent with C_{50} carotenoids were also identified in the smear-ripened cheese varieties Maroilles, Epoisses, and Mont d'Or (Galaup et al., 2007). Further efforts were made by Giuffrida et al. (Giuffrida et al., 2015) to identify these compounds synthesized by one of these bacteria derived from the smear-ripened cheese, *Glutamicibacter arilaitensis*. These authors reported the co-occurrence of decaprenoxanthin and sarcinaxanthin, as identified via UV-Visible spectra, and MS precursor and high molecular weight MS/MS product ions extracted from this bacterial species (Giuffrida et al., 2015). *Micrococcus luteus*, a halophile, has also been reported to produce sarcinaxanthin (Netzer et al., 2010). These bacteria are abundant in the rind of smear ripened cheeses (Bockelmann, 2002), either sourced from brine, old-young smear, or from the house microbiota. Saprenoxanthin has the same base

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structure as the symmetric decaprenoxanthin and sarcinaxanthin, but with one ε-ring and one γ-ring. Genes related to the production of these three C_{50} carotenoids were identified in *C. glutamicum* (Netzer et al., 2010), a reason to suspect the presence of saprenoxanthin in these extracts. A mass and spectra consistent with flavuxanthin, an intermediate in the biosynthesis of sarcinaxanthin, was also observed (Netzer et al., 2010). Collectively, the identification of C_{50} carotenoids in all four cheeses suggest that the microbiome developed on the surface of these cheese as part of smear is likely contributing to the development of these carotenoids.

Brevibacterium linens is usually added as an adjunct secondary culture during Limburger and Taleggio manufacture, but we did not identify any C_{40} carotenoids uniquely produced by this bacterium in these cheese extracts. Echinenone (tentatively identified in the core and rind) and lycopene (in the rind only) were believed to be the only C_{40} carotenoids of bacterial origin identified in all cheeses. *Dietzia sp.*, known to be present on the surface of smear-ripened cheeses, produces echinenone, and can also produce the C_{50} carotenoid called "C.p. 450" and the C_{40} carotenoid canthaxanthin (absent from our mixture). The other C_{40} carotenes and xanthophylls observed are likely to be sourced from milk, as only mutant strains of bacteria have been found to produce significant concentrations of these species. Lycopene is an intermediate in the biosynthetic pathway of almost all carotenoids, including decaprenoxanthin and sarcinaxanthin, hence its origin in bacteria that produce these carotenoids is highly likely (Krubasik et al., 2001; Netzer et al., 2010). Thus, our data suggests that not only the end products of the pathways play a role in colour development of the smear cheese surface, but also the intermediates. We also screened for and did not find other carotenoids anticipated in these samples, i.e. phytoene, phytofluene, neurosporene, bacterioruberin, bisanhydrobacterioruberin, torularhodin, torulene (produced by coryneform bacteria), and agelaxanthin (from *Brevibacterium*) (Giuffrida et al., 2020).

Identifying the carotenoids of smear-ripened cheeses helps us to determine their contribution to provitamin A carotenoid delivered in the human diet. Indeed, the bioavailability of carotenoids is higher when consumed in conjunction with lipid-rich foods (Unlu, Bohn, Clinton, & Schwartz, 2005), including dairy lipids (Goltz, Campbell, Chichumroonchokchai, Failla, & Ferruzzi, 2012). Furthermore, research has suggested that co-consuming lipid with a provitamin A rich meal aids in conversion to vitamin A in the intestine of humans who are considered "low converters" (Kopec et al., 2014). Additionally, C_{50} carotenoids are found sparingly in the human diet, and these results demonstrate that they may be obtained from this family of cheeses. The novel C_{50} structure has strong antioxidant properties (Miller, Sampson, Candeias, Bramley, & Rice-Evans, 1996; Naguib, 2000), and could serve as a skin protectant, either through dietary means or through applications in sunscreens and other light protecting cosmetics (Heider et al., 2014; Osawa et al., 2010). Their advantages in human health have not been extensively studied, and this area is ripe for further work.

5. Conclusions

To our knowledge, this is the first detailed study of carotenoids present in Ashbrook, Charloe, Limburger, and Taleggio cheese extracts. The provitamin A carotenoid β-carotene was the dominant species in all samples tested, followed by α-carotene and minor quantities

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of epoxy-β-carotene, and β-cryptoxanthin. Rind samples specifically contained carotenoids which reflect a red hue, including lycopene and the C50 bacterial carotenoids decaprenoxanthin and sarcinaxanthin. These bacterial carotenoids are plausibly synthesized by coryneform bacteria such as *C. glutamicum*, *G. arilaitensis*, etc., and their interactions with other microbes, casein hydrolysates, and the cheese environment may lead to the development of these rich surface colours classically associated with smear-ripened cheeses. Future research on these cheeses should focus on quantitative analysis, as well as further identification of minor species in the absence of the cheese matrix (i.e., by testing biological isolates grown independent of the cheese matrix).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

DAD	diode array detector
MS	mass spectrometry
MTBE	methyl *tert*-butyl ether
QTof	quadrupole time-of-flight mass spectrometer
MS/MS	tandem mass spectrometry
UHPLC	ultra-high pressure liquid chromatography

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Highlights

- Carotenoids from four smear-ripened cheeses were extracted and profiled.
- Provitamin A carotenoids predominated in both rind and core samples
- Lycopene and C50 bacterial carotenoids were unique to rind samples
- Similar carotenoids profiles were observed between all smear-ripened cheeses tested

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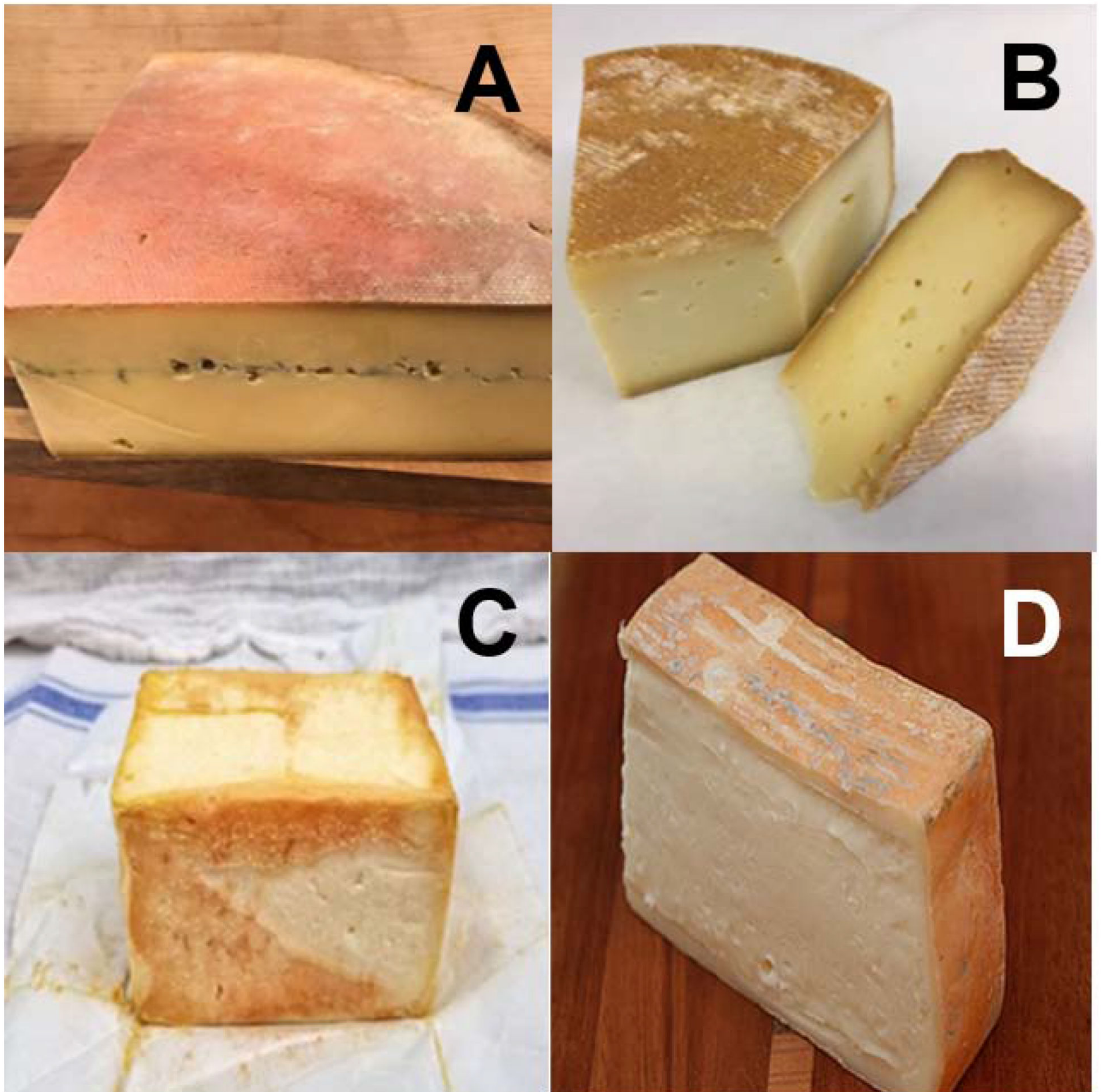

Figure 1.

Photos of the smear-ripened cheeses investigated: (A) Ashbrook, (B) Charloe, (C) Limberger, (D) Taleggio

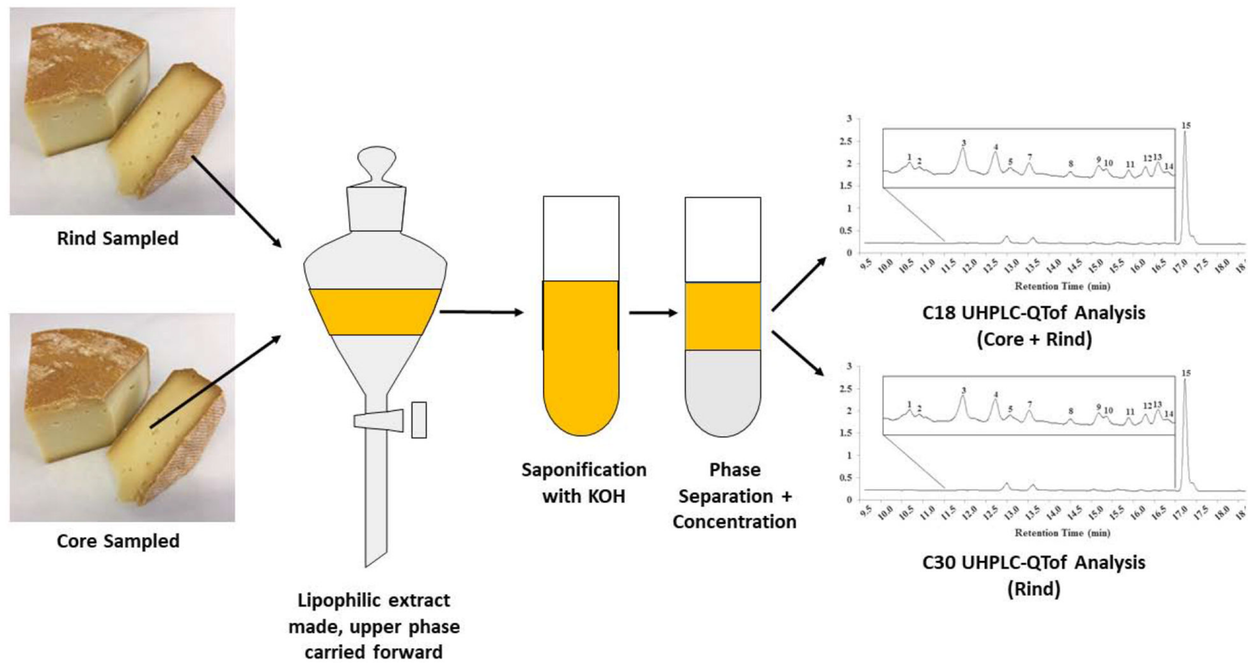

Figure 2.

Experimental scheme employed to study carotenoids in the rind and core of smear-ripened cheeses

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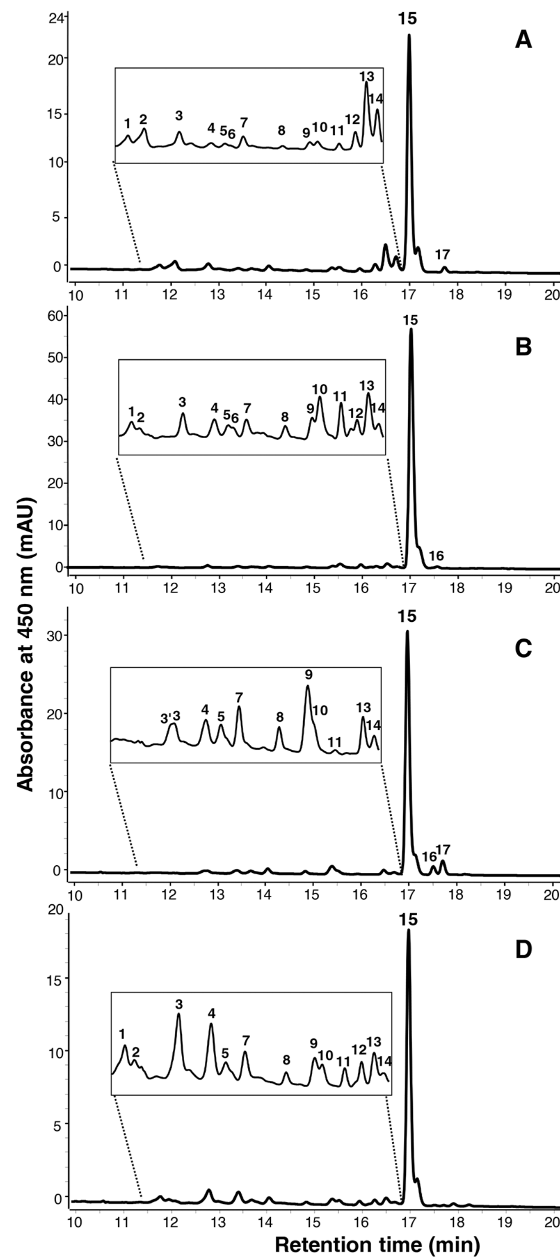

Figure 3.

UHPLC-DAD chromatogram at 450 nm of non-polar rind extracts following saponification and concentration, as separated on a C18 column. (A) Ashbrook, (B) Charloe, (C) Limberger, (D) Taleggio. Peak numbers correspond to carotenoids noted in Table 1.

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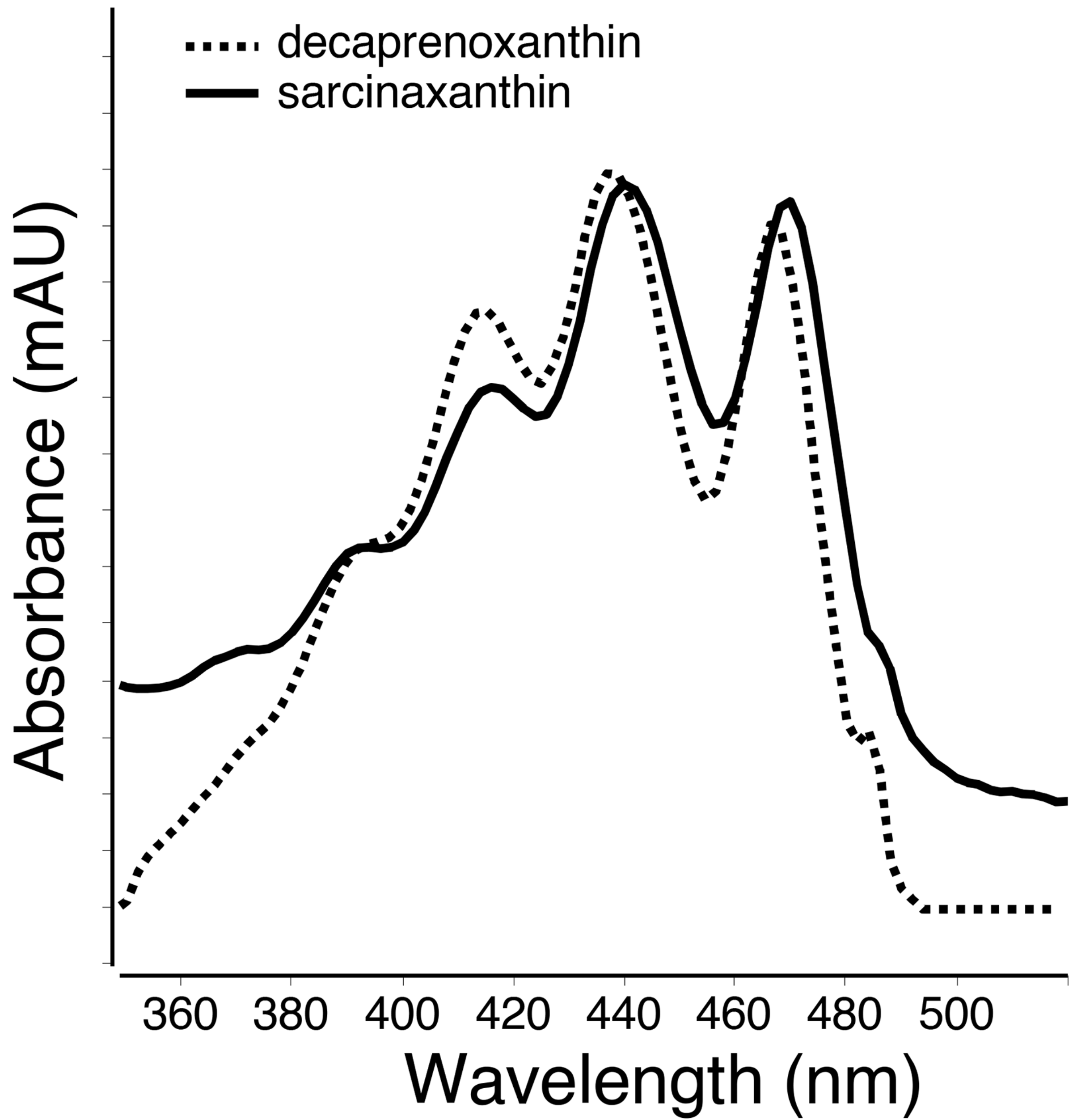

Figure 4.
UV-Vis spectra of decaprenoxanthin (dashed line) and sarcinoxanthin (solid line) observed in the cheese rind extract.

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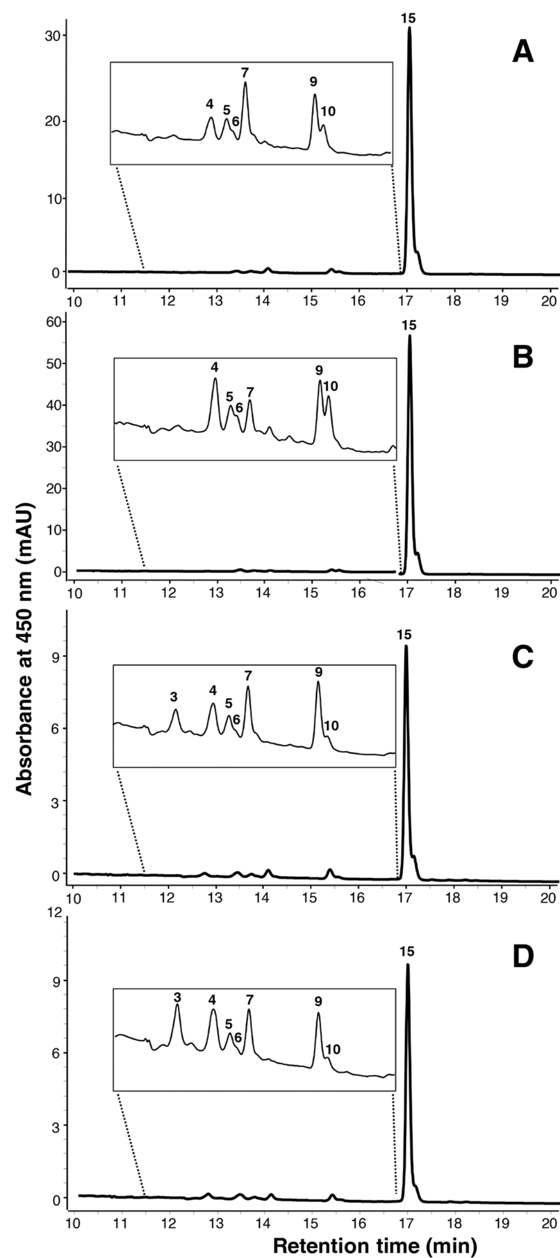

Figure 5.

UHPLC-DAD chromatogram at 450 nm of non-polar core extracts following saponification and concentration, as separated on a C18 column. (A) Ashbrook, (B) Charloe, (C) Limberger, (D) Taleggio. Peak numbers correspond to carotenoids noted in Table 1.

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Table 1.

Carotenoids identified in core and rind extracts from smear-ripened cheeses.

Peak[a]	RT (min)	Compound	UV-Vis spectra (nm)	Precursor Ion(s) (m/z)[c]	Precursor ion species	Product ions (m/z) produced with collision energy = 10 V			
						Ashbrook (A)	Charloe (C)	Limburger (L)	Taleggio (T)
1	11.7	decaprenoxanthin[f,i]	414,438[b], 470	705.5602 687.5569	$[M+H]^+$ $[M+H-H_2O]^+$	669.6, 595.4, 613.5	669.6, 595.4		669.6, 595.4, 547.5
2	11.9	sarcinaxanthin[f,i]	416,440[b], 470	705.5602 687.6606	$[M+H]^+$ $[M+H-H_2O]^+$	669.4, 595.4, 613.3	669.6		669.6
2'	12.0	*cis*-sarcinaxanthin[f,i]	416, 440[b], 470	687.6572	$[M+H]^+$	669.4, 595.4, 473.1	-	-	-
3',[d]	12.70	unknown	420,440[b], 478 (C,L)	569.4650	$[M+H]^+$		ND	551.5, 459.3, 205.15, 313.2	
3	12.74	unknown	458 round spectra (A,T)	ND	ND	ND			ND
4[d]	13.4	β-cryptoxanthin[e,j]	422, 452[b], 474	553.4398 535.4283	$[M+H]^+$ $[M-H_2O+H]^+$	535.4	ND	135.1	461.2, 135.1
5[d]	13.6	*cis*-β-cryptoxanthin[f,j]	426, 450[b], 474	553.4393 535.4278	$[M+H]^+$ $[M-H_2O+H]^+$	535.4, 497.3, 135.1	535.4, 497.2, 135.1	535.4, 497.2	535.45, 497.2
6[d]	13.7	unknown	444[b], 472 (C,A,T); 452 round spectra (L)	ND	ND	ND	ND	ND	ND
7[d]	14.2	echinenone[f,i]	468[b]	551.4229	$[M+H]^+$	203.1, 495.2, 255.2, 459.3	203.1, 495.2	203.1, 495.2, 255.2	203.1, 495.2, 255.2
8	15.0	sarcinaxanthin or saprenoxanthin ester[f,i]	412, 436[b], 468	705.5602	$[M+H-FA]^+$	687.7	ND	687.4	ND
9[d]	15.6	mono-epoxy-β-carotene[f,j]	402, 428[b], 458	553.4370	$[M+H]^+$	535.4,461.3, 205.1, 177.1	535.4, 461.3, 205.1, 177.1	535.4, 461.3, 205.1, 177.1	535.4, 461.3, 205.1,177.1
10[d]	15.7	α-cryptoxanthin ester[g,e,j]	420, 446[b], 472	553.4359 535.5011	$[M+H-FA]^+$ $[M-H_2O-FA+H]^+$	ND	ND	ND	ND
11	16.2	β-cryptoxanthin ester[g,j]	422, 452[b], 476	553.4955 535.4895	$[M+H-FA]^+$ $[M-H_2O-FA+H]^+$	ND	ND	ND	ND
12	16.5	*cis*-lycopene tentatively co-eluting with 1lavuxanthin or decaprenoxanthin ester[g,i]	414, 438, 470[b], 500 (A,C,T)	537.4438 705.5602	$[M+H]^+$ $[M+H-FA]^+$	ND	ND		ND
13	16.7	all-*trans*-lycopene[e,i]	444, 472[b], 504	537.4382	$[M+H]^+$	457.3, 413.3, 119.0, 177.1	ND	457.3, 177.1	537.4

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Peak[a]	RT (min)	Compound	UV-Vis spectra (nm)	Precursor Ion(s) (m/z)[c]	Precursor ion species	Product ions (m/z) produced with collision energy = 10 V			
						Ashbrook (A)	Charloe (C)	Limburger (L)	Taleggio (T)
14	16.9	*cis*-lycopene[e,i]	342[h] 360[h] 440, 466[b], 494	537.4382	$[M+H]^+$	413.3,177.1, 119.0	ND	ND	ND
15[d]	17.2	carotene (both β- and α-[e,j])	424, 452[b], 478	537.4382	$[M+H]^+$	457.3, 445.3, 413.3, 255.2, 177.1, 137.1	457.3, 413.3, 177.1, 137.1	457.3, 413.3, 255.2, 177.1, 137.1	457.3, 413.3, 255.2, 177.1, 137.1
16	17.7	decaprenoxanthin or sarcinaxanthin ester coeluting with another species[g,i]	470[b] (C,L)	705.5602 255.2324	$[M+H-FA]^+$ $[FA]^-$		ND	ND	
17	17.9	decaprenoxanthin or sarcinaxanthin ester coeluting with anotherspecies[g,i]	285, 476[b] (A,L)	705.5602	$[M+H-FA]^+$	ND		ND	

[a]Corresponds to peak numbers used in Figures 2 and 3

[b]Denotes λ_{max}

[c]The primary precursor ion detected using atmospheric pressure chemical ionization (APCI) operated in positive ion mode

[d]Carotenoids also identified in cheese cores

[e]Identity confirmed with authentic standards (i.e. retention time, UV-Vis spectra, MS and MS/MS spectra consistent with standard)

[f]Retention order, UV-Vis spectra, and MS precursor, product ions consistent with literature

[g]Available information (retention order and/or UV-Vis spectra and/or MS precursor) consistent with literature

[h]*cis* peak.

[i]carotenoids sourced from bacteria

[j]carotenoids sourced from milk

Abbreviations: A = Ashbrook, C = Charloe, L = Limberger, ND = not detected, due to lack of signal, or presence of co-eluting species interfering with spectra, RT = retention time, T = Taleggio