Stability of vegetable microconstituents at intermediate temperatures : fate of vitamins and other micro-components in products based on fruits and vegetables

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Stability of vegetable micro-constituents at intermediate temperatures: fate of vitamins and other micro-components in products based on fruits and vegetables

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OPTIFEL food for elderly

INRA SCIENCE & IMPACT

To my wonderful grandmother Gertraud Gsuk

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AA: Ascorbic acid

AIC: Akaike Information Criterion

AR: Average Requirement

5-CH_3-H_4folate: 5-Methyltetrahydrofolate

CTCPA: Centre Technique de la Conservation des Produits Agricoles

DAD: Diode Array Detector

DHA: Dehydroascorbic acid

DTT: Dithiothreitol

DFE: Dietary Food Equivalent

E_A: Activation Energy

EDTA: Ethylenediaminetetraacetic acid

EFSA: European Food Safety Authority

HPLC: High Performance Liquid Chromatograhy

LC-MS: Liquid Chromatography-Mass Spectrometry

MES: 2-(N-morpholino)-ethanesulfonic acid hydrate

OPD: *ortho*-phenylendiamine

OPTIFEL: Optimized Products for Elderly Populations

PreSens: Precision Sensing GmbH

PRI: Population Reference Intake

PTFE: Polytetrafluoroethylene

PVDF: Polyvinylidene fluoride

R^2: Coefficient of Determination

SIDA: Stable Isotopic Dilution Assay

UL: Tolerable Upper Intake Level

USDA: United States Department for Agriculture

Résumé

Dans le cadre du projet européen « Optimized Products for Elderly Populations (OPTIFEL) » (ou « produits optimisés pour des gens âgés »), des produits alimentaires sont conçus pour les besoins particuliers des personnes âgées. Puisque cette population est souvent mal-nourrie, l'objective du projet consistait à produire des aliments riches en nutriments et appétants. Ce but a été mis en œuvre en enrichissant des produits à base de fruits et légumes avec des protéines, des minéraux et vitamines, dont la vitamine C et les folates. Cependant, les deux dernières vitamines sont connues pour être fragiles et pour être rapidement perdues lors du chauffage. Pour atteindre le but de la supplémentation, c'est-à-dire augmenter l'absorption des nutriments, l'étude de leur stabilité est d'une grande importance. Ce travail, en particulier, a été dédié à l'étude de la stabilité de la vitamine C et des folates lors du réchauffage des aliments.

Le réchauffage des aliments nécessite de respecter une température minimum de 60°C afin d'éviter la croissance des bactéries sporulées. Une deuxième contrainte, qui se démarque des méthodes de cuisson, est la durée du maintien en température. Selon que le réchauffage se déroule à la maison ou dans un système de restauration collective en liaison chaude, le temps de réchauffage est de courte durée ou peut atteindre quelques heures.

La pomme et la carotte ont été choisies en tant qu'exemple d'un fruit et un légume pour le projet OPTIFEL et aussi pour le travail présent. La pomme et la carotte sont des produits qui sont appréciés à travers l'Europe et contiennent des quantités naturelles négligeables en vitamine C et folates.

La stabilité de la vitamine C a fait objet de nombreuses études dans la littérature. Cependant, les facteurs qui impactent sa stabilité ont été principalement examinés en solution modèle et leur importance respective dans un vrai aliment manque d'études. Bien que la disponibilité de l'oxygène ait un impact primordial, et qu'il soit connu que l'oxygène est soluble jusqu'à 100°C, sa disponibilité dans le milieu alimentaire est très mal connue pendant le chauffage à des températures intermédiaires. L'acide folique est un vitamère synthétique, qui est habituellement utilisé pour la supplémentation mais qui a l'inconvénient de pouvoir masquer un déficit en vitamine B_{12}. C'est pourquoi le vitamère naturellement abondant, l'acide 5-méthyltétrahydrofolate, a été proposé comme alternative pour

l'enrichissement. Son inconvénient majeur, outre le prix, est qu'il est fragile et se dégrade rapidement en l'absence de réducteurs.

L'objectif de cette thèse de doctorat consistait à comprendre la stabilité de la vitamine C et de l'acide 5-méthyltétrafolique à des températures intermédiaires. Une attention particulière a été portée à la stabilité dans des matrices alimentaires et à la disponibilité de l'oxygène. Dans un premier temps, la stabilité de la vitamine C et de l'acide 5-méthyltétrahydrofolique a été étudiée à une échelle laboratoire. Ensuite, l'impact des différentes méthodes de réchauffage a été examiné. Le travail a été divisé en quatre chapitres. Le premier chapitre a été consacré à l'étude de la stabilité de la vitamine C. Dans le deuxième chapitre, la disponibilité de l'oxygène a été étudiée. La troisième étude a été dédiée à la stabilité de l'acide 5-méthyltétrahydrofolique. Et dans le quatrième chapitre, trois méthodes de réchauffage ont été comparées.

Dans le premier chapitre, l'impact des différents facteurs intrinsèques (pH, concentration initiale de l'acide ascorbique, matrice alimentaire) et extrinsèques (température de 40 à 80°C, hauteur de remplissage des tubes) sur la stabilité de la vitamine C ont été comparés. Dans ce but, un modèle a été cherché qui s'ajuste à toutes les cinétiques. Avoir un modèle commun est en effet indispensable pour pouvoir comparer quantitativement la vitesse de la dégradation dans les différentes conditions. Puisque l'acide ascorbique et l'acide déshydroascorbique (le dernier est le premier produit d'oxydation de l'acide ascorbique) sont tous les deux, bioactifs, leur somme a été prise en compte dans la modélisation. Dans les conditions standard de cette étude (80°C, 3 mmol/kg acide ascorbique, volume de remplissage 1,5 mL), l'ajustement aux points expérimentaux du modèle d'ordre zéro a été meilleur qu'avec un modèle de premier ordre, tel que classiquement décrit pour la dégradation de la vitamine C dans les milieux modèles.

Les facteurs intrinsèques ont eu un effet faible à négligeable. La vitamine C est légèrement moins stable dans la purée de pomme (pH 3,5) que dans la purée de carotte (pH 5,5). En conséquence, les deux matrices peuvent être utilisées de manière interchangeable pour la supplémentation. De plus, le pH n'a influencé la vitesse de dégradation qu'en solution modèle (tampon citrate-phosphate) et pas

dans le sérum de purée de pomme. Un changement de la concentration initiale de l'acide ascorbique, nécessaire par exemple si on veut adapter la quantité aux besoins de différents groupes de consommateurs, est facile à contrôler puisque la vitesse de dégradation est indépendante de la concentration et de la durée: la quantité perdue par intervalle de temps reste constante, une caractéristique des modèles d'ordre zéro.

Par contre, la stabilité de la vitamine C est fortement influencée par des facteurs extrinsèques. L'effet de la température est majeur entre 40 et 60°C. Cependant, entre 60-80°C, une zone qui est cruciale en liaison chaude, les constantes de vitesse ne varient pas avec la température, probablement à cause d'une compétition entre la diminution de la disponibilité de l'oxygène, second substrat de la réaction et l'effet d'accélération des réactions lié à l'augmentation de l'énergie disponible. Ainsi, en chauffant les aliments à 80°C, la marge de sécurité est agrandie et en même temps la valeur nutritionnelle reste la même qu'en chauffant à 60°C. L'impact de la hauteur de remplissage des tubes a été associé au ratio surface/volume et ainsi à la disponibilité de l'oxygène puisque les autres facteurs sont restés constants. C'est pourquoi la disponibilité de l'oxygène pendant le chauffage dans différentes matrices a été étudiée dans l'étude suivante.

Dans le deuxième chapitre, la disponibilité de l'oxygène dans différentes matrices a été examinée pendant 8 h à 80°C et a été reliée à la perte en vitamine C. Le comportement de l'oxygène en solution modèle a été fondamentalement différent de celui dans les matrices alimentaires.

Dans de l'eau pure contenant 3 mmol kg^{-1} d'acide ascorbique, la teneur de l'oxygène diminue d'abord légèrement puis augmente de nouveau au cours de temps, tandis qu'une fraction de l'acide ascorbique est transformée en acide déshydroascorbique. Cependant, la vitamine C totale, c'est-à-dire la somme de l'acide ascorbique et de l'acide déshydroascorbique, ne diminue pas. Il semble qu'un initiateur manquait pour la dégradation de la vitamine C. En présence des ions Fe^{3+} ou quand l'acide ascorbique a été dissous dans un tampon citrate-phosphate, le comportement de l'oxygène a été similaire que dans l'eau. La teneur en oxygène augmente de nouveau après une diminution initiale. Pourtant, dans ces milieux, la vitamine C a été perdue après le chauffage.

Dans la compote de pomme et la purée de carotte, l'oxygène a été consommé entièrement en moins d'une heure. Il n'a pas été remplacé dans le milieu au cours de temps par l'oxygène venant de l'espace de tête. Cette baisse montre l'importance de l'oxygène de l'espace de tête. L'oxydation d'autres molécules semble mener à une consommation accélérée de l'oxygène. Pourtant, la vitamine C est quand même dégradée. Lorsque des ions Fe^{3+} ont été ajoutés à la compote de pomme, l'acide déshydroascorbique a été rapidement formé, avant même le traitement thermique, mais la dégradation de la vitamine C pendant le chauffage n'a pas été accélérée. Une baisse de température de 80°C à 70°C ou 60°C, n'a pas influencé l'étendue de la dégradation dans de l'eau contentant des ions ferriques, mais a stabilisé entièrement la vitamine C dans la compote de pomme.

En absence d'oxygène, la vitamine C était stable dans de l'eau contenant des ions Fe^{3+} et dans le sérum de compote de pomme. Il semble que l'oxygène plus un initiateur doivent tout les deux être présents pour déclencher la dégradation de la vitamine C. De plus, l'hydrolyse de l'acide déshydroascorbique apparaît être dépendante de l'oxygène. Une conversion partielle de l'acide ascorbique en acide déshydroascorbique a eu lieu déjà pendant la substitution de l'oxygène par l'azote dans de l'eau contentant des ions ferriques. Cependant, aucune perte de vitamine C n'a été constatée après le traitement du milieu en anaérobie. Il est possible que l'hydrolyse de l'acide déshydroascorbique soit initiée par des dérivés actifs de l'oxygène qui sont seulement formés dans des milieux en aérobie.

Le troisième chapitre a été consacré à l'étude de la stabilité de l'acide 5-méthyltétrahydrofolique. Les effets de la matrice alimentaire, de la température (60-80°C) et de la concentration en vitamine C ont été étudiés. L'acide 5-méthyltétrahydrofolique a rapidement disparu, y compris dans des matrices alimentaires qui contiennent des antioxydants. Il est dégradé complètement en moins d'une demie heure dans du tampon, la compote de pomme et la purée de carotte. Les matrices alimentaires n'ont pas pu stabiliser l'acide 5-méthyltétrahydrofolique. Par contre l'ajout de l'acide ascorbique s'est montré efficace pour prévenir cette dégradation. En augmentant la quantité d'acide ascorbique ajouté (10-50 mg/100 g), la période de protection a pu être prolongée. Lorsque une concentration de 50 mg/100 g est utilisée, l'acide 5-méthyltétrahydrofolique est stable pendant 3 h à 80°C. Cependant,

quand la quantité initiale est de 10 mg/100 g ou 25 mg/100 g, l'acide ascorbique, bien qu'encore présent en excès par rapport à la concentration de l'acide 5-méthyltétrahydrofolique ou la teneur en oxygène, une dégradation de l'acide 5-méthyltétrahydrofolique s'amorce avant la fin des 3 h. Un facteur dépendant de la concentration initiale en acide ascorbique et du temps semble donc intervenir. Une baisse de la température de 80°C à 60°C ne permet pas de diminuer la dégradation de l'acide 5-méthyltétrahydrofolique.

En termes de stabilité, un enrichissement simultané en vitamine C (50 mg/100 g) et en acide 5-méthyltétrahydrofolique permet d'avoir une supplémentation aussi stable au cours du réchauffage qu'avec l'acide folique.

Ainsi, dans le quatrième chapitre, l'acide 5-méthyltétrahydrofolique et l'acide ascorbique ont été utilisés simultanément pour enrichir des purées de pomme et de carotte. L'impact de trois méthodes de réchauffage a été étudié, donc deux qui sont utilisées dans les ménages (Micro-onde et Actifry ®) et une pour garder les aliments en liaison chaude en restauration collective. Le chauffage au micro-onde jusqu'à ébullition a duré 1,5 minutes pour une portion de 250 g et en utilisant l'Actifry ®, environ 20 minutes était nécessaires pour atteindre 80°C avec 1 kg de purée. Le temps de chauffage à 80°C en bain-marie des purées préchauffées a été fixé à 3 h. Dans ces conditions, la vitamine C et l'acide 5-méthyltétrahydrofolique n'ont pas été perdus ou de façon négligeable. L'impact de la hauteur de remplissage qui a été révélé à l'échelle laboratoire n'a pas pu être validé. De plus, aucune différence entre les prélèvements près de la surface et au fond des récipients n'a pu être constatée.

Pour le projet OPTIFEL, la stabilité de la vitamine C et l'acide 5-méthyltétrahydrofolique pendant le réchauffage dans des conditions réelles est une bonne nouvelle. La quantité qui est supplémentée est préservée dans les produits même si les produits sont gardés à chaud pendant 3 h à 80°C. Leur quantité initiale ne doit pas être adaptée et n'a pas besoin d'être modélisée. Cette conclusion est inattendue, puisque les deux vitamines sont d'habitude décrites comme étant fragiles à la chaleur et l'oxygène. Ce travail a confirmé le fait que leur stabilité dépend de nombreux facteurs. De plus, il a été démontré que les conditions en solution modèles ne sont pas comparables avec celles des matrices alimentaires,

surtout en termes de disponibilité de l'oxygène. Les facteurs influençant la stabilité des deux vitamines sont complexes, et elle doit donc toujours être testée dans les conditions réelles.

Zusammenfassung

Im Rahmen des europäischen Projekts „Optimized Products for Elderly Populations (OPTIFEL)“ (oder „optimierte Produkte für ältere Populationen“), werden Nahrungsmittel für die speziellen Bedürfnisse von älteren Menschen entwickelt. Da dieser Teil der Bevölkerung oft unter Fehlernährung leidet, bestand das Ziel der Studie darin, schmackhafte Lebensmittel mit hohem Nährstoffgehalt herzustellen. Produkte auf Früchte- und Gemüsebasis wurden deshalb mit Proteinen, Mineralien und Vitaminen, darunter Vitamin C und Folate, angereichert. Die zwei letzteren Vitamine sind jedoch bekannt dafür sehr empfindlich zu sein und schnell während Erwärmungen verloren zu gehen. Um dennoch das Ziel der Supplementierung zu erreichen, welches darin besteht die Nährstoffmenge zu erhöhen, ist die Studie ihrer Stabilität von großer Bedeutung. Die vorliegende Arbeit widmete sich im Besonderen der Stabilität von Vitamin C und Folaten während der Wiederaufwärmung von Lebensmittel.

Wenn Lebensmittel warm gehalten werden ist eine minimale Temperatur von 60°C einzuhalten, um das Wachstum von sporenbildenden Bakterien zu verhindern. Eine zweite Einschränkung, die sich von Kochmethoden abgrenzt, ist die Länge der Wärmebehandlung. Je nachdem, ob die Aufwärmung in Haushalten oder in Großküchen stattfindet, ist die Zeitspanne von kurzer Dauer oder kann bis zu einige Stunden betragen.

Äpfel und Karotten wurden als Frucht- und Gemüsebeispiel für das Projekt OPTIFEL und auch für die vorliegende Arbeit gewählt. Äpfel und Karotten sind Produkte die in ganz Europa geschätzt werden und die zu vernachlässigende, natürliche Mengen an Vitamin C und Folaten enthalten.

Die Stabilität von Vitamin C wurden in zahlreichen Studien in der Literatur untersucht. Jedoch wurden die Faktoren welche die Stabilität beeinflussen, hauptsächlich in Modellösungen erforscht und ihre jeweilige Bedeutung in einem richtigen Lebensmittel ist nicht bekannt. Obwohl Sauerstoff einen entscheidenden Einfluss hat und es bekannt ist, dass Sauerstoff bis 100°C löslich ist, ist seine Verfügbarkeit in Lebensmittel während Erwärmungen bei mittleren Temperaturen unbekannt. Folsäure ist ein synthetisches Vitamer, welches gewöhnlich für Supplementierungen verwendet wird aber in Verdacht steht, ein Defizit an Vitamin B_{12} zu maskieren. Aus diesem Grund wurde das in der

Natur dominierende Vitamer, 5-Methyltetrahydrofolat, als Alternative für Anreicherungen vorgeschlagen. Es ist allerdings sehr empfindlich und degradiert schnell in Abwesenheit von Reduktionsmittel.

Das Ziel der Doktorarbeit bestand darin, die Stabilität von Vitamin C und 5-Methyltetrahydrofolate im mittleren Temperaturbereich zu verstehen. Spezielle Aufmerksamkeit wurde der Stabilität in Lebensmittelmatrizen und der Verfügbarkeit von Sauerstoff gewidmet. Zunächst, wurde die Stabilität von Vitamin C und 5-Methyltetrahydrofolat im Labormaβstab untersucht. Anschließend wurde der Einfluss von verschiedenen Methoden die für die Wiederaufwärmung von Lebensmittel genutzt werden erforscht. Die Arbeit wurde in vier Kapitel aufgeteilt. Das erste Kapitel wurde der Stabilitätsstudie von Vitamin C gewidmet. Im zweiten Kapitel wurde die Sauerstoffverfügbarkeit, im dritten Kapitel die Stabilität von 5-Methyltetrahydrofolat und im vierten Kapitel der Einfluss von drei Aufwärmmethoden untersucht.

Im ersten Kapitel wurden die Einflüsse von verschiedenen intrinsischen Faktoren (pH, Anfangskonzentration von Ascorbinsäure, Lebensmittelmatrix) und extrinsischen Faktoren (Temperatur, 40-80°C, Füllhöhe von Behältern) auf die Stabilität von Vitamin C verglichen. Dafür wurde zunächst ein Modell gesucht, das alle Kinetiken gut beschreibt. Das Ziel eines gemeinsamen Modells war es, die Stabilität unter allen Degradierungsbedingungen quantitativ vergleichen zu können. Da Ascorbinsäure und Dehydroascorbinsäure (letztere ist das erste Oxidationsprodukt von Ascorbinsäure) beide bioaktiv sind, wurde ihre Summe bei der Modellierung berücksichtigt. Unter Standardbedingungen der Studie (80°C, 3 mmol/kg Ascorbinsäure, 1,5 mL Füllvolumen) passte sich das Modell nullter Ordnung besser an die experimentellen Werten an als das Modell erster Ordnung. Letzteres wird üblicherweise verwendet um die Degradierung von Vitamin C in Modellösungen zu beschreiben.

Die untersuchten intrinsischen Faktoren hatten einen kleinen bis zu vernachlässigenden Einfluss. Vitamin C war in Apfelmus (pH 3,5) geringfügig instabiler als in Karottenpüree (pH 5,5). Folglich sind beide Matrizen für die Supplementierung geeignet. Desweitern beeinflusste der pH die

Degradierung nur in Modellösung (Citrat-Phosphat-Puffer) und nicht in Apfelmus-Serum. Eine Änderung der Anfangskonzentration von Ascorbinsäure welche z.B. notwendig ist, wenn die angereicherte Menge an die speziellen Bedürfnisse von verschiedenen Konsumentengruppen angepasst werden soll, ist leicht zu kontrollieren, da die Geschwindigkeit der Degradierung unabhängig von der Konzentration und der Zeit ist. Die Menge an Ascorbinsäure die pro Zeitintervall degradierte, war konstant.

Hingegen wurde die Stabilität von Vitamin C stark von extrinsischen Faktoren beeinflusst. Der Temperatureffekt war groß im Bereich 40-60°C. Zwischen 60-80°C, eine Zone die von entscheidender Bedeutung für die Wiederaufwärmung von Lebensmittel ist, variierten die Geschwindigkeitskonstanten jedoch nicht mit der Temperatur. Dies liegt wahrscheinlich daran, dass der Sauerstoffgehalt (Sauerstoff ist das zweite Substrat der Reaktion) abnahm und durch den Mangel, die Reaktion nicht mehr durch eine Zunahme der Temperatur bzw. der Energie beschleunigt wurde. Dies bedeutet, dass wenn Lebensmittel bei 80°C erhitzt werden, das Risiko mikrobiellen Wachstums sinkt und gleichzeitig derselbe Nährwert wie bei 60°C beibehalten wird. Der Einfluss der Füllhöhe von Versuchsröhrchen wurde dem Verhältnis Oberfläche/Volumen zugeschrieben und damit der Sauerstoffverfügbarkeit, da alle anderen Faktoren konstant blieben. Die Sauerstoffverfügbarkeit im Medium während der Erwärmung wurde deshalb in der folgenden Studie untersucht.

Im zweiten Kapitel wurde der Sauerstoffgehalt während acht Stunden bei 80°C in verschiedenen Matrizen verfolgt, und die Auswirkung auf den Vitamin C Gehalt analysiert. Das Sauerstoffverhalten in den Modellösungen war im Vergleich zu den Lebensmittelmatrizen stark unterschiedlich.

In Wasser in dem 3 mmol/kg Ascorbinsäure gelöst war nahm der Sauerstoffgehalt am Anfang leicht ab und stieg im Laufe der Zeit wieder an. Obwohl am Ende ein Teil der Ascorbinsäure als Dehydroascorbinsäure vorlag, ging Vitamin C insgesamt, d.h. die Summe aus Ascorbinsäure und Dehydroascorbinsäure, nicht verloren. Für die Degradierung von Vitamin C schien ein Initiator zu fehlen. In Anwesenheit von Fe^{3+} Ionen und auch als Ascorbinsäure in einem Citrat-Phosphat Puffer gelöst wurde war das Sauerstoffverhalten dem in Wasser sehr ähnlich. Nach einer anfänglich, kurzen

Abnahme stieg die Sauerstoffmenge wieder an. Vitamin C wurde jedoch in diesen Medien zerstört. Fe^{3+} Ionen bzw. eine Komponente im Citrat-Phosphat Puffer schien diesmal die Degradierung auszulösen.

In Apfelmus und Karottenpüree wurde Sauerstoff in weniger als einer Stunde konsumiert. Der Sauerstoffgehalt wurde zu keinem messbaren Ausmaß durch Sauerstoff im Luftraum im Laufe der Zeit ersetzt. Durch die komplette Abnahme im Medium gewinnt Sauerstoff im Luftraum in diesen Matrizen an Bedeutung. Obwohl die Oxidation von anderen Molekülen zu einem beschleunigten Sauerstoffverbrauch zu führen schien, wurde Vitamin C dennoch zerstört.
Als Fe^{3+} Ionen zu Apfelmus zugeben wurden, wurde Dehydroascorbinsäure bereits vor der Wärmebehandlung schnell gebildet. Die Degradierung von Vitamin C wurde jedoch nicht beschleunigt. Eine Temperaturabnahme von 80°C nach 70°C oder 60°C hat das Ausmaß der Degradierung in Wasser mit Fe^{3+} Ionen nicht beeinflusst, aber die Degradierung in Apfelmus komplett verhindert, weshalb vermutet werden kann, dass sich die Mechanismen in den Medien unterscheiden.

In Abwesenheit von Sauerstoff war Vitamin C in Wasser mit Fe^{3+} Ionen und auch in Apfelmus-Serum stabil. Sauerstoff und ein Initiator scheinen gemeinsam anwesend sein zu müssen um die Degradierung von Vitamin C auszulösen. Zudem hing die Hydrolyse von Dehydroascorbinsäure von Sauerstoff ab. In Wasser mit Fe^{3+} Ionen wurde Ascorbinsäure zu Dehydroascorbinsäure, bereits während der Substituierung von Sauerstoff durch Stickstoff, zu einem großen Teil umgewandelt. Nach der Wärmebehandlung des anaeroben Mediums wurde allerdings kein Verlust von Vitamin C festgestellt. Es ist möglich, dass die Hydrolyse von Dehydroascorbinsäure von reaktiven Sauerstoffspezies ausgelöst wird, welche nur in aeroben Medien gebildet werden.

Das dritte Kapitel wurde der Stabilität von 5-Methyltetrahydrofolat gewidmet. Der Einfluss der Lebensmittelmatrix, der Temperatur (60-80°C) und der Vitamin C Konzentration wurde untersucht. 5-Methyltetrahydrofolat ging in Modellösung und auch in den untersuchten Lebensmittelmatrizen, die Antioxidantien enthalten und dadurch einen stabilisierenden Effekt haben könnten, schnell verloren. 5-

Methyltetrahydrofolat in Ameisensäure-Puffer, Apfelmus oder Karottenpüree, degradierte in weniger als einer halben Stunde. Nur die Zugabe von Ascorbinsäure führte zu einer wirksamen Stabilisierung.

Die Zeitspanne in der 5-Methyltetrahydrofolat stabil ist, konnte durch eine größere Menge an zugebener Ascorbinsäure (10-50 mg/100 g) verlängert werden. Als 50 mg/100 g Ascorbinsäure verwendet wurde, konnte 5-Methyltetrahydrofolate während drei Stunden bei 80°C komplett stabilisiert werden. Als jedoch der Anfangsgehalt 10 mg/100 g oder 25 mg/100 g betrug, war die Menge an Ascorbinsäure noch im molaren Überschuss im Vergleich zu der Konzentration an 5-Methyltetrahydrofolat oder Sauerstoff, als die Degradierung begann. Ein Faktor, welcher von der Anfangskonzentration von Ascorbinsäure und der Zeit abhängig war, schien zu intervenieren. Eine Abnahme der Temperatur von 80°C nach 60°C führte nicht zur Stabilisierung von 5-Methyltetrahydrofolat.

Was die Stabilität angeht, ist 5-Methyltetrahydrofolat bei gleichzeitiger Anreicherung von 50 mg/100 g Ascorbinsäure ebenso gut für Supplementierungen geeignet wie Folsäure. Aus diesem Grund wurden Apfelmus und Karottenpüree im vierten Kapitel mit 5-Methyltetrahydrofolat und Ascorbinsäure angereichert. Der Einfluss von drei Methoden die zur Aufwärmung von Lebensmittel verwendet werden wurde untersucht. Darunter waren zwei Methoden, die in Haushalten angewendet werden (Mikrowelle und Actifry ®) und eine um Lebensmittel in Großküchen warmzuhalten. Die Erhitzung bis zum Siedepunkt von 250 g Püree dauerte 1.5 Minuten mit der Mikrowelle, und mit dem Actifry ® Gerät waren 20 Minuten nötig, bis das Püree (1 kg) 80°C erreicht hatte. Die Erhitzungszeit im Wasserbad bei 80°C von vorgewärmtem Püree betrug drei Stunden. Unter diesen Bedingungen degradierten Vitamin C und 5-Methyltetrahydrofolat nicht bzw. zu einem vernachlässigenden Teil. Der Einfluss der Füllhöhe welcher im Labormaßstab festgestellt wurde, konnte nicht validiert werden. Des Weiteren wurde kein Stabilitätsunterschied an der Oberfläche und am Boden von Behältern festgestellt.

Für das Projekt OPTIFEL ist die Stabilität von Vitamin C und 5-Methyltetrahydrofolat während der Wiederaufwärmung von Lebensmittel eine gute Neuigkeit. Die Menge die supplementiert wird, wird

in den Produkten erhalten auch wenn diese drei Stunden bei 80°C warmgehalten werden. Ihre Anfangskonzentration muss nicht angepasst werden und Verluste müssen nicht modelliert werden. Dies wurde nicht erwartet da beide Vitamine für ihre Hitze- und Sauerstoffempfindlichkeit bekannt sind. Die Arbeit hat bestätigt, dass ihre Stabilität von vielen Faktoren abhängt. Des Weiteren wurde gezeigt, dass Bedingungen in Modellösungen, insbesondere was die Sauerstoffverfügbarkeit betrifft, nicht mit denen in Lebensmittelmatrizen vergleichbar sind. Faktoren, die die Stabilität beeinflussen sind komplex, weshalb ihre Stabilität immer unter realen Bedingungen getestet werden sollte.

Introduction

The European Union included 507 million people in 2014 (EuroStat, 2014). One fifth was aged 65 or older. Until 2080, this population will rise to one third and the ratio of elderlies who will be 80 or older is predicted to double (Figure 1).

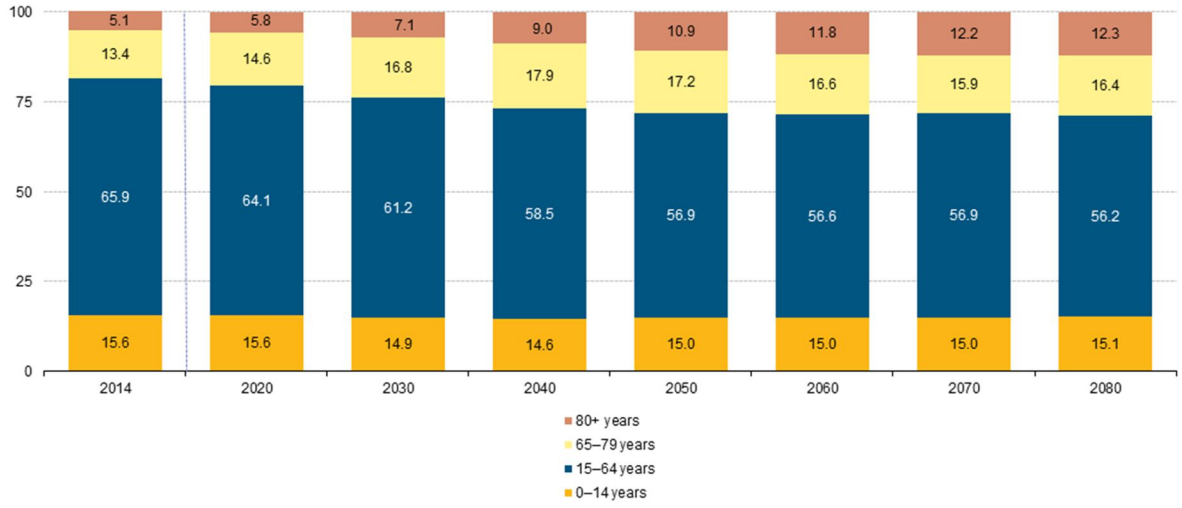


Figure 1: Demographic change in Europe from 2014 until 2080.

Population quotas (in %) of four age groups are illustrated.

The graphic was taken from EuroStat (2014).

Many elderlies suffer from malnutrition which influences crucially their "health, well-being and autonomy" (Di Francesco et al., 2007). In the study of Paker-Eichelkraut et al. (2013), inadequate intake of vitamin C, folates and other micronutrients of more than half of nursing home residents in Germany, was ascribed to the loss of eating pleasure and rejection of food. The European Project "Optimized Food Products for Elderly Populations" (OPTIFEL), coordinated by Dr. Catherine Renard, aims to improve elderlies' nutritional status by conceiving food products for their special needs. To cope with malnutrition, products based on vegetables and fruits will be enriched with proteins, calcium, folates, vitamin C, D, E and B_{12}. The project runtime comprises 3.5 years and will end in 2017. A funding of three million euros is provided by the 7th framework program of the European Union. 26 European partners are involved.

The project OPTIFEL includes six work packages which work on exigencies of food products addressed to seniors, to increase their nutritional value and acceptability. Work package 1 examined food preferences of elderlies by respecting cultural aspects in different European countries. The maximal force which can be applied by seniors to open packages as well as the necessary minimal font size of labels was examined. Consumer acceptances of different sugar/acid ratios were tested, and delivery costs of food products were calculated. Work package 2 reviewed nutritional recommendations which were inserted in a "food calculator" that is a software that estimates individual nutritional needs. Texture requirements and the sensory change of aged people were investigated. Work package 3 tested the feasibility of new products which were based on fruits and vegetables. Apples and carrots were the main models for technological and scientific developments. Work package 4, in which the present work was involved, was dedicated to the evolution of products during storage and reprocessing which included aspects of food sensory and nutritional quality, and microbial safety of products. Work package 5 worked on the "product acceptance at homes and nursing homes" and Work package 6 addressed the "dissemination and technology transfer" of OPTIFEL. A newsletter was regularly diffused and a website was established where news, published articles and congress participations were announced.

Vitamin C and folates are known to be fragile molecules. To comply with the intention of vitamin enrichment that is enhancing their supply, knowledge about their stability is fundamental. In the present work, the stability of vitamin C and 5-methyltetrahydrofolate under reheating conditions was studied. Results are presented in the form of papers which were already published, accepted for publication or are in preparation for submission. An overview of papers is given at the end of this paragraph.

The first chapter was dedicated to a literature review about the stability of ascorbic acid and the folate vitamer 5-methyltetrahydrofolate. Both subchapters are organized in the same way. Firstly, general characteristics of the vitamin namely physical and chemical properties, its biosynthesis, health effects, average requirements, the bioavailability and vitamin status in Europe are described. In connection

follows the main part treating the vitamin stability and its influences, degradation products, the degradation mechanism and the impact of domestic processing methods.

In the second chapter, the objective and general approach is described. The lack of knowledge is summarized and the research questions and hypotheses are pointed out.

Since a thesis by publications was chosen as presentation form, principal information about the used materials and methods is given within each publication in the chapter "Results". The independent chapter "Material and Methods" which follows in connection to the general approach is supplementary and devoted to the principles of used analytical methods and detailed experimental protocols.

The chapter "Result" is divided into four main parts corresponding to four publications. The first three studies address factors that impact the stability of the two vitamins on a laboratory scale with the aim to better understand their degradation. The information was designed to be used in the fourth and last study, to optimize vitamin losses on a scale-up level.

The first study, in particular, was devoted to the stability of vitamin C in a real food product. Intrinsic factors that is pH, concentration and the food matrix, as well as extrinsic factors, namely temperature and filling height, were studied in a quantitative way. A fundamental impact of the availability of oxygen was revealed, which led to the second chapter. Oxygen contents during heat treatments at an intermediate temperature range (60-80°C) in different media were studied. Kinetics within the food matrix at different positions, and in the headspace were compared. Factors were investigated which potentially may impact the equilibrium between headspace and dissolved oxygen, and thus the degradation of vitamin C. Studied factors comprised the food matrix, Fe^{3+} ions and temperature. Furthermore, the combination of known degradation impacts was studied as degradation was only initiated when several, crucial factors were conjointly present. Initial and final vitamin C concentrations were analyzed to link them to the respective oxygen availability.

In the third study, the stability of 5-methyltetrahydrofolate was compared with that of folic acid. The latter is a synthetic folate vitamer that is commonly used for supplementation and is known for its high stability. Ways to increase the stability of 5-methyltetrahydrofolate were studied and included the

effect of temperature, the food matrix and different vitamin C concentrations. The work leading to this chapter was carried out at the Chair of Analytical Food Chemistry at the Technical University of Munich, headed by the co-supervisor of this work, Professor Rychlik.

And finally, in the last chapter, the effect of three warming-up methods on the stability of vitamin C and 5-methyltetrahydrofolate in apple purée and carrot purée was examined. The impact of a microwave and an Actifry ® treatment, which are warming-up methods that can be encountered at people's homes, and a warm holding device which is usually used in canteen kitchens, was tested.

The thesis finishes with a general conclusion of main findings and propositions for future research.

Oral presentations within the "OPTIFEL" framework were held in Hämeenlinna/Finland, Paris/France and Girona/Spain.

The work has led to the following articles that were already published, were accepted or are in preparation for submission:

Delchier, N., Herbig, A.-L., Rychlik, M. and Renard, C.M.G.C. (2016), Folates in Fruits and Vegetables: Contents, Processing, and Stability. *Comprehensive Reviews in Food Science and Food Safety*, 15: 506–528, (published).

Herbig, A.-L., Renard, C.M.G.C. Factors that impact the stability of vitamin C at intermediate temperatures in a food matrix. *Food Chemistry*, (accepted).

Herbig, A.-L., Maingonnat, J.-F., Renard, C.M.G.C. Oxygen availability in model solutions and purées during heat treatment and the impact on vitamin C degradation. *LWT-Food Science and Technology,* (accepted).

Herbig, A.-L., Delchier, N., Striegel, L., Rychlik, M., Renard, C.M.G.C. Stability of 5-methyltetrahydrofolate supplemented to apple purée and carrot purée, (in preparation for submission).

Herbig, A.-L., Moustiés, C., Renard, C.M.G.C. Impact of three warming up methods on the stability of vitamin C and 5-methyltetrahydrofolate in apple and carrot purée, (in preparation for submission).

Herbig, A.-L., Renard, C.M.G.C. Factors that impact the stability of ascorbic acid and its degradation mechanism, (in preparation for submission).

Two posters were presented at international conferences namely on the "XVIII Euro Food Chem" in Madrid/Spain and on the "Euro-Mediterranean Symposium for Fruit and Vegetable Processing" in Avignon/France. The posters were entitled as follows:

Herbig, A.-L., Renard, C.M.G.C. Factors that impact vitamin C's stability in apple purée, *XVIII Euro Food Chem*, 13-16 October 2016, Madrid, Spain

Herbig, A.-L., Maingonnat, J.-F., Renard, C.M.G.C. Oxygen availability at intermediate temperatures and the impact on vitamin C degradation, *Euro-Mediterranean Symposium for Fruit and Vegetable Processing*, 4-6 April 2016, Avignon, France

Literature Review: Vitamin C

This chapter is planned to be submitted as a review in a food science journal. It will be entitled "Factors that impact the stability of ascorbic acid and its degradation mechanism". It starts with general information about vitamin C. In connection follows a paragraph about factors that impact its stability and a paragraph about degradation products and the degradation mechanism. The chapter ends with the influence of domestic processing.

1. Introduction

Vitamin C is commonly known for its beneficial health effects and its high susceptibility to heat. It is usually referred exclusively to ascorbic acid (Figure 2) however its oxidized form, dehydroascorbic acid, is reconverted to ascorbic acid in the gastrointestinal tract and many cells and is thus contributing to the vitamin activity. Besides, dehydroascorbic acid exhibits also anti-oxidative properties (Deutsch, 2000; Jaffe, 1984; Wilson, 2002).

The vitamin property is finally lost when dehydroascorbic acid is hydrolyzed irreversibly to 2,3-diketo-L-gulonic acid (Figure 2). In aqueous, liquid systems (water activity higher than 0.980), the prevalent degradation pathway proceeds via the oxidation of ascorbic acid. The oxidation reaction of ascorbic acid to dehydroascorbic acid is the rate limiting step as the transformation of dehydroascorbic acid to 2,3-diketo-L-gulonic acid proceeds rapidly (Van Bree et al., 2012).

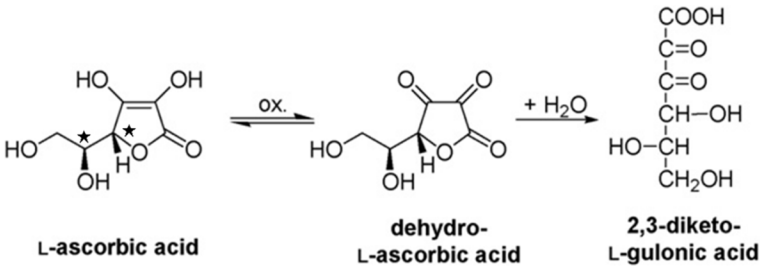


Figure 2: Degradation of L-ascorbic acid.

⋆ stereo centers

Taken from Schulz, Trage, Schwarz, and Kroh (2007), adapted version.

The stability of ascorbic acid depends however on numerous factors. The higher the water activity (a_w) or the moisture content, the faster the deterioration of ascorbic acid (Lee & Labuza, 1975). As at intermediate temperatures, the aerobic degradation pathway is predominant, the exposition to oxygen is crucial for the degradation of vitamin C (Dhuique-Mayer et al., 2007; Oey, Verlinde, Hendrickx, & Van Loey, 2006; Verbeyst, Bogaerts, Van der Plancken, Hendrickx, & Van Loey, 2013). With rising temperature the quantity of dissolved oxygen decreases, but simultaneously the supply of energy rises (Penicaud, Peyron, Gontard, & Guillard, 2012). A lot of factors that impact the degradation of vitamin C, were only studied in model solutions and their importance in real food matrices is lacking (Aka et al., 2013; Kaack & Austed, 1998; Lee & Labuza, 1975; Oey et al., 2006; Rojas & Gerschenson, 1997, 2001; Wilson, Beezer, & Mitchell, 1995; Yamauchi, Nimura, & Kinoshita, 1993). The availability of oxygen during heat treatments is not known so far but might be different in foods compared to model solutions as also other food constituents may consume oxygen. Besides, food components as polyphenols interact with vitamin C as a consequence of redox reactions (Bradshaw, Barril, Clark, Prenzler, & Scollary, 2011). Transition metal ions and light activate oxygen and decrease thus the stability of ascorbic acid (Choe & Min, 2005). Many models have been applied to describe the depletion of ascorbic acid and range from linear, exponential to biphasic and the Weibull model, indicating that the degradation behavior depends strongly on the used experimental conditions (Dhuique-Mayer et al., 2007; Eisonperchonok & Downes, 1982; Johnson, Braddock, & Chen, 1995; Kennedy, Rivera, Lloyd, Warner, & Jumel, 1992; Oey et al., 2006; Rojas & Gerschenson, 2001; Sapei & Hwa, 2014; Van den Broeck, Ludikhuyze, Weemaes, Van Loey, & Hendrickxx, 1998). Besides, a quantitative overview of degradation products is missing up to date. This can be assumed to be due to the numerous derivatives that are formed during degradation and their respective chemical and physical properties necessitating several detection methods. And finally, a general recommendation of heating technologies cannot be given as the outcome depends a lot on the experimental set-up.

Latest reviews are based on the behavior of L-ascorbic acid in plants and in a wine environment (Bradshaw et al., 2011; Davey et al., 2000). Given the large number of factors that impact the stability of vitamin C and their interactions during heat treatments at intermediate temperatures, a profound

knowledge on a general basis is however necessary before optimizing heat treatments protocols. This chapter starts with general information about vitamin C, followed by a paragraph about factors that impact stability in model solutions and food products. In connection, models that can be found in literature to describe the degradation pace of vitamin C are presented. An overview of degradation products is given with the respective detections constraints, and the degradation mechanism is illustrated. And finally, the impact of different cooking methods is presented.

2. General information

2.1 Physical and chemical characteristics

The structure of ascorbic acid is sugar related. Ascorbic acid is the aldono-1,4-lactone of a hexonic acid with an endiol group between C-2 and C-3 (Davey et al., 2000). It exhibits two stereo centers indicated in Figure 2 by stars. Ascorbic acid has two pK_a values, the first at 4.3 and a second at 11.8 (Bradshaw et al., 2011). L-ascorbic acid exhibits a standard redox potential of + 0.35 V (Matsui, Kitagawa, Okumura, & Shigeta, 2015). It is a hydrophilic molecule and thus little soluble in lipophilic media. Its solubility in water accounts to 0.33 g/mL and in ethanol (95%) to 0.033 g/mL (Jaffe, 1984). L-ascorbic acid is the predominant form in food products (Behrens & Madere, 1994).

2.2 Sources

Acerola, green chili peppers and black currents contain high amounts of vitamin C namely 1678 mg/100 g, 243 mg/100 g and 181 mg/100 g (USDA, 21.7.2017). The stereoisomer D-erythorbic acid, also known as D-isoascorbic acid or D-arabosacorbic acid, is allowed as food additive by the European Food Safety Authority (EFSA, 2013). The antiscorbutic activity of D-erythorbic acid in guinea pig is weak to insignificant (Goldman, Gould, & Munro, 1981; Reiff & Free, 1959) but it is more effective than ascorbic acid in preventing colds (Clegg & Macdonald, 1975). Ascorbic acid can be synthesized by plants and many animals but not by humans (Arrigoni & De Tullio, 2002). The biosynthesis in plants starts with GDP-D-mannose and in animals with UDP-D-glucuronic acid. In

humans, the gene encoding the enzyme L-gulono lactone oxidase, which is necessary for the last step of the biosynthesis of ascorbic acid, is inactive. Because humans cannot produce ascorbic acid, it is an essential micronutrient and indispensable to maintain a good health status for many reasons.

2.3 Health effects and daily requirements

It has an antiscorbutic effect, increases iron absorption, is an important antioxidant and has a preventive effect on cancer formation (Baker et al., 1971; Block, 1991; Cook & Monsen, 1977; Peterkofsky, 1991). Its role in cells might be exclusively related to its reducing capacity and inactivation of reactive radicals but not to a direct involvement in catalytic cycles (Padh, 1990).

An Average Requirement (AR) of 90 mg/day for men and of 80 mg/day for women was set by the European Food Safety Authority (EFSA) aiming to main a plasma concentration of 50 µmol/L by taking into account metabolic vitamin losses, an absorption of 80 % and an urinary excretion of 25 % (EFSA, 2013). A Population Reference Intake (PRI) was inferred from the AR respecting a coefficient of variation (CV) of 10 %. A PRI of 110 mg/day for men and of 95 mg/day for women has thus been proposed. A Tolerable Upper Intake Level (UL) for vitamin C was not fixed by the EFSA due to lack of data and to the low toxicity reported by available studies. Levine et al. (1996) and also Frei, Birlouez-Aragon, and Lykkesfeldt (2012) suggested a higher optimum of the dietary intake namely of 200 mg/day. They point out that recommendations are usually based on the amount to prevent scurvy but should be higher as an increased intake of vitamin C has also other health benefits.

2.4 Bioavailability

Vitamin C is completely bioavailable when 200 mg are ingested in a single dose. The extent of the bioavailable amount declines when a dose of 500 mg or higher is absorbed (Levine et al., 1996). The excretion of oxalate and urate is only increased at a daily dose of 1000 mg. Ascorbic acid and dehydroascorbic acid are absorbed by enterocytes and renal epithelial cells in the lumen of the intestine and renal tubes (Wilson, 2005). Ascorbic acid is actively transported via the transporters SVCT1 and SVCT2 by a Na^+-dependent gradient (Tsukaguchi et al., 1999). Even if the two

transporter isomers are structurally and functionally related, they are found in different tissues; SVCT1 in epithelial systems, that is in the intestine, kidney and liver, and SVCT2 in metabolically active cells and specialized tissues of the brain, eye and other organs. Active transport is regulated, and impaired by age or in the case of disease. Smoking causes also less absorption of vitamin C (Schectman, Byrd, & Gruchow, 1989). The transport of ascorbic acid by non-facilitated diffusion occurs to a negligible extent (Wilson, 2005). Dehydroascorbic acid can be absorbed by GLUT type transporters (Liang, Johnson, & Jarvis, 2001), however under physiological conditions that is in the presence of glucose, this absorption way is assumed to play a minor role.

2.5 Vitamin C status in Europe

The vitamin C status in Europe ranges from a mean intake of women of 81 mg/d in Poland to 152 mg/d in Germany, and for men from 81 mg/d in France to 152 mg/d in Germany. Only around 3 % of people in Europe ingest less than the lower reference intake (Mensink et al., 2013). The mean intake of people that are over 60 years old is lower compared to people below 60 years. By supplementation, the intake increases by approximately 20-30 mg/d (Schectman et al., 1989).

2.6 Industrial production and applications

The Reichstein-Grüssner process, which dates from 1933, was the predominant industrial process to generate vitamin C for a long time. D-sorbitol was hereby converted via 2-keto-L-gulonic acid to L-ascorbic acid by *Gluconobacter oxydans* and several chemical conversions. Since recently, *Ketogulonicigenium vulgare* is used as biocatalyst (Pappenberger & Hohmann, 2014).

Ascorbic acid is often used as quality indicator (Megias-Perez, Gamboa-Santos, Cristina Soria, Villamiel, & Montilla, 2014; Romeu-Nadal, Castellote, Gaya, & Lopez-Sabater, 2008) due to its oxygen and heat susceptibility. Furthermore, it is employed in the food industry to avoid enzymatic browning of fruits and vegetables, as oxygen scavenger to protect beer against oxidation reactions and linked off flavors, as color and flavor stabilizer of cured meat, to ameliorate the quality of dough, and

in wines as reduction agent (Bauernfeind, 1982; Soliva-Fortuny & Martin-Belloso, 2003; Vermeiren, Devlieghere, van Beest, de Kruijf, & Debevere, 1999).

3. Stability impacts

3.1 Temperature

Ascorbic acid is known to be a heat- and oxygen-sensitive molecule (Garcia-Torres, Ponagandla, Rouseff, Goodrich-Schneider, & Reyes-De-Corcuera, 2009). Ascorbic acid degrades during storage on a week time scale in citrus juice concentrates (Burdurlu, Koca, & Karadeniz, 2006; Polydera, Stoforos, & Taoukis, 2003). When temperature rises up to 100°C, ascorbic acid is lost within hours or minutes depending on the initial concentration (Dhuique-Mayer et al., 2007; Oey et al., 2006). Temperature sensitivity is usually described by the Arrhenius law providing the activation energy (E_A), an empirical value. In model solution, Rojas and Gerschenson (2001) determined activation energies between 54 kJ/mol and 63 kJ/mol (24-90°C) depending on the used model composition that is the type of sugar, sugar alcohol or salt type. An activation energy of 36 kJ/mol (50-100°C) was determined for citrus juice by Dhuique-Mayer et al. (2007) and of 39 kJ/mol (20-45°C) for orange juice by Manso, Oliveira, Oliveira, and Frias (2001). At intermediate temperatures that is up to 100°C, oxygen is still soluble in water (Penicaud et al., 2012). Increasing temperature enhances on the one hand the supply of energy and on the other hand decreases the amount of oxygen that is soluble and thus available for oxidations.

3.2 Dissolved oxygen

Ascorbic acid degrades via an oxidative and a non-oxidative degradation pathway (Schulz et al., 2007; Yuan & Chen, 1998a). The loss of ascorbic acid at an intermediate temperature range can be significantly reduced when oxygen is removed since the oxidative pathway is predominant (Dhuique-Mayer et al., 2007; Garcia-Torres et al., 2009). However, the vitamin C activity is only lost when dehydroascorbic acid is hydrolyzed to 2,3-diketogulonic acid, a fast reaction. The oxidation step, that

is the reaction of ascorbic acid to dehydroascorbic acid, is the rate limiting step (Van Bree et al., 2012).

Factors that influence the solubility of oxygen are the polarity of the medium, temperature, salinity, and the °Brix (Chaix, Guillaume, & Guillard, 2014; Penicaud et al., 2012). Oxygen is soluble up to 100°C in water, and is more soluble in fats than in aqueous solutions. With increasing temperature, salinity and °Brix, that is presence of other soluble solids, dissolved oxygen contents decrease. Oxygen concentrations are so far only known before and after, but not during heat treatments (Garcia-Torres et al., 2009). The oxygen amount after heat treatment is usually lower than the initial one what can be linked to oxygen consumption as a consequence of oxidation reactions (Garcia-Torres et al., 2009). When working without headspace, an initial, rapid ascorbic acid degradation is followed by a stagnation period in strawberry and raspberry paste which are heated up to 80°C (Verbeyst et al., 2013). The plateau of kinetics was assumed to be the consequence of complete dissolved oxygen consumption which was however not measured.

A dynamic equilibrium exists between oxygen dissolved in the medium and oxygen in the headspace (Van Bree et al., 2012). During oxidation reactions, dissolved oxygen is consumed and replaced by oxygen from the headspace. When the oxygen concentration in the headspace is high, degradation of ascorbic acid is accelerated. When oxygen in the headspace is depleted, oxygen values in the headspace increase initially and then decrease again. At the beginning, the lack in the headspace is adjusted through oxygen coming from the medium, later the equilibrium is shifted again as a result of oxygen consumption within the medium. The diffusion of oxygen is in general faster in aqueous media compared to oils (Penicaud, Guilbert, Peyron, Gontard, & Guillard, 2010). Oxygen diffusion rates decrease in the following order: water, agar, orange juice, mashed apple, olive oil, mashed tomato. Diffusion measurements in food matrices are delicate due to concomitant occurring oxidation reactions which can be circumvented by prior pre-oxidation of the media (Penicaud et al., 2010). Diffusion coefficients are not impacted by an increase of the viscosity of lipid media (Chaix, Guillaume, Gontard, & Guillard, 2016). The diffusion of oxygen at 20°C is slow and thus significant

oxidation occurs only near the surface of agar gel (Penicaud, Broyart, Peyron, Gontard, & Guillard, 2011).

Methods to remove oxygen range from vacuum-deaeration, gas substitution, membrane deaerators to enzyme-based deprivation and oxygen scavengers (Garcia-Torres et al., 2009). Under certain conditions, as it is the case when food is reheated in a domestic environment, oxygen removal is however rather difficult to realize and oxygen will inevitably be encountered. More knowledge of the availability of oxygen in real products during heat treatments is necessary to improve preservation of ascorbic acid in systems containing oxygen. The elucidation of the exact role of oxygen that is to know if catalytic or quantitative molar amounts are involved in the degradation of ascorbic acid, is important. Methods to quantify oxygen were recently reviewed by Penicaud et al. (2012). Instant measurement of dissolved and gaseous oxygen is nowadays possible by oxygen sensors which are based on a luminescence signal. The luminophore of the sensor is excited by light and the remission of luminescence that is the intensity or time decay is measured by the detector. Molecular oxygen quenches the fluorescence and reduces thus the measured signal.

3.3 Reactive oxygen species (ROS)

Ascorbic acid is well known to be a strong antioxidant. It scavenges radicals and regenerates other antioxidants such as polyphenols, carotenoids or tocopherol (Bradshaw et al., 2011; Young & Lowe, 2001). The oxidation of ascorbic acid proceeds via a radical intermediate. Finally, two electrons are released resulting in dehydroascorbic acid (Davey et al., 2000). Triplet oxygen is the most stable and predominant form of oxygen (Choe & Min, 2005). Two electrons of triplet oxygen have parallel spins and are located in anti-bonding orbitals classifying triplet oxygen as a diradical. It thus predominantly reacts with radicals. Reactive oxygen species, that is hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$) and hydroxyl- ($HO^{\cdot}$), peroxy- ($ROO^{\cdot}$), alkoxy- ($RO^{\cdot}$) and hydroperoxy- ($HOO^{\cdot}$) radicals are formed by chemical and enzymatic reaction, irradiation, but also during the degradation of ascorbic acid (Boatright, 2016; Choe & Min, 2005).

A considerable amount of hydrogen peroxide, that is up to 32.5 µmol/L from 100 µmol/L ascorbic acid, was measured by Boatright (2016) during the oxidation of ascorbic acid leading to an auto-acceleration of its degradation. Hydrogen peroxide undergoes significant decomposition when pH is over 9, at temperatures > 23°C (Qiang, Chang, & Huang, 2002) and also by exposition to light or Fe^{2+} ions (Choe & Min, 2005). Hydroxyl radicals that are more reactive than hydrogen peroxide are formed during the decomposition. The standard redox potential of L-ascorbic acid is + 0.35 V (Matsui et al., 2015) and hence lower than that of hydroxyl radicals which is of + 2.31 V (Choe & Min, 2005). Other reactive oxygen species such as singlet oxygen or superoxide anions also react with ascorbic acid.

In a food matrix, not only ascorbic acid but also major food components such as sugars or amino acid exhibit high reaction rates with reactive oxygen species (Choe & Min, 2005). As there are usually present in much higher amounts than vitamins, their destruction is however proportionally less important than that of ascorbic acid. The enzyme catalase transforms H_2O_2 into oxygen and water, and the enzyme superoxide dismutase inactivates superoxide. Both enzymes decelerate thus the degradation of ascorbic acid (Boatright, 2016; Miyake, Kim, & Kurata, 1999). Protection of ascorbic acid by enzymes is however not of lasting nature at elevated temperatures as they are destroyed rapidly (Sapers & Nickerson, 1962; Walker, McLellan, & Robinson, 1987).

Reactive oxygen species are predominantly quantified via a fluorescent signal. The agent dichlorodihydrofluorescein diacetate (DCFH-DA) is therefore often used which is oxidized by reactive oxygen species to dichlorofluorescein emitting fluorescence. Limitations of this method are reviewed by Kalyanaraman et al. (2012). Concisely, as the method is unspecific, a main drawback is the overestimation of the signal for example by the presence of Fe^{2+} ions. The importance of reactive oxygen species for oxidation reactions in foods can so far only be assumed as no measurement method exists.

3.4 Light

Singlet oxygen is formed in foods in the presence of photosensitizers and the irradiation of light (Min & Boff, 2002). Photosensitizers absorb light energy which is then transmitted to triplet oxygen being

excited and thus transformed to singlet oxygen. Examples for photosensitizers are chlorophyll, riboflavin but also synthetic colorants. The reaction rate of singlet oxygen is much higher than that of triplet oxygen as its activation energy is lower. Ascorbic acid is susceptible to singlet oxygen; 1 mmol/L of ascorbic acid degrades within 4 hours in contrast to a glucose loss of only 2 µmol/L after 8 hours, when dissolved in phosphate buffer (0.1 mol/L) that contains lens proteins as photosensitizer and when irradiated with UVA light (λ = 338 nm) (Giangiacomo, Olesen, & Ortwerth, 1996). Under these conditions, 40 % of ascorbic acid is degraded by singlet oxygen and 24 % by superoxide anion. H_2O_2 exhibits only a negligible impact in the presence of singlet oxygen. However, it is activated by Fe^{3+} ions during irradiation (Zepp, Faust, & Hoigne, 1992). Fe^{3+} ions are reduced by light to Fe^{2+} ions which, in turn, produces very reactive hydroxyl radicals from H_2O_2.

The effect of amino acids is ambivalent since it depends strongly on the present photosensitizer (Jung, Kim, & Kim, 1995). Cysteine exhibits an antioxidant effect on ascorbic acid when the photosensitizer is riboflavin or methylene blue. Alanine and phenylalanine have a protective effect when riboflavin is present but act, in turn, as pro-oxidants when methylene blue is the photosensitizer of the system.

3.5 Metals

Vitamin C is stable in the absence of transition metals at room temperature during 15 minutes (Buettner, 1988). Adding Fe^{3+} ions to an aqueous solution, increases not only the reaction of ascorbic acid to dehydroascorbic acid, but also that of dehydroascorbic acid to 2,3-diketogulonic acid (Figure 3) (Serpen & Gökmen, 2007). Since ascorbic acid is almost completely preserved in a model solution containing Cu^{2+} ions under anaerobic conditions, the catalytic activity of metals might however not be a simple redox reaction with ascorbic acid being oxidized and the metal ion being reduced, but an interaction of ascorbic acid, oxygen and the metal ion (Boatright, 2016).

Although the hydrolysis of dehydroascorbic acid is also influenced by Fe^{3+} ions, this is not the result of an oxidation reaction as its oxidative state does not change. Fe^{2+} ions and H_2O_2 are generated during the oxidation of ascorbic acid (Boatright, 2016). An interaction of reactive hydroxyl ions that are

formed when Fe^{2+} ions react with H_2O_2 might explain the effect of Fe^{3+} ions on the hydrolysis of dehydroascorbic acid (Choe & Min, 2005).

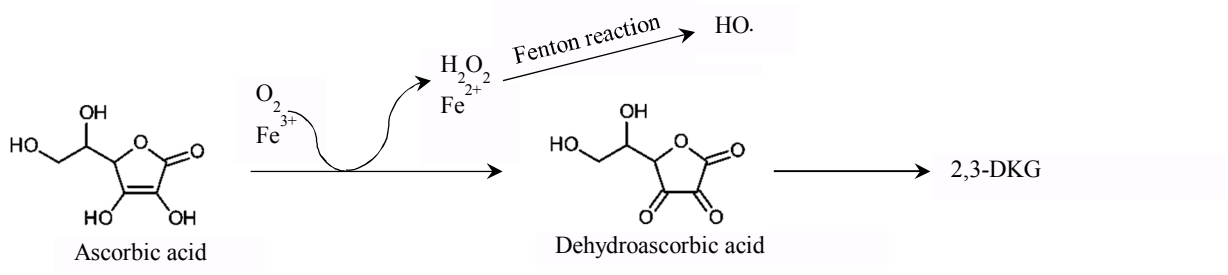


Figure 3: Degradation of ascorbic acid in the presence of Fe^{3+} ions.

The coordination of ascorbic acid with transition metals has been reviewed by Zumreoglu-Karan (2006). They point out that although ascorbic acid has many donor atoms that could build complexes with metals, complexes are primarily formed with oxygen in position O-3, or in position O-3 and O-2 (Figure 4).

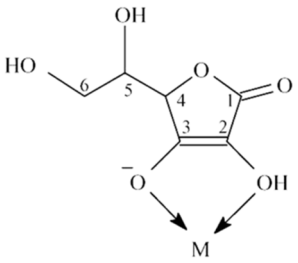


Figure 4: Chelate coordination of ascorbic acid in its anionic form.

Taken from Zumreoglu-Karan (2006).

Chelators have an effect on the oxidation of ascorbic acid since they influence the reactivity of metals. Khan and Martell (1967a) established an order for the catalytic activity of copper and iron in the presence of different chelators. The catalytic activity is the strongest when copper is used in the form iminodiacetatocopper (II) with a rate constant of 0.30×10^2 M^{-1} sec^{-1}, pH 2, 25°C) and the lowest when it is present as ethylendiaminetetracetatocuprate (II) with a rate constant of 0.09×10^1 M^{-1} sec^{-1}, pH 2, 25°C). In terms of Fe^{3+} ions, the strongest effect was recorded for 2-

hydroxyethyliminodiacetatoiron (III) (0.9×10^2 M^{-1} sec^{-1}, pH 2, 25°C) and the lowest for diethylenetriaminepentacetatoferrate (III) (0.8×10^1 M^{-1} sec^{-1}, pH 2, 25°C). NaCl protects ascorbic acid by preventing the interaction of transition metals ions with oxygen (Harel, 1994). However, in the presence of a strong ligand, the effect of NaCl is negligible. Furthermore, NaCl has no impact on the interaction of iron or copper with H_2O_2.

pH also impacts the interaction of ascorbic acid with metals. The anionic form of ascorbic acid is more susceptible to Cu^{2+} than Fe^{3+} ions; its neutral form in contrast, is more prone to Fe^{3+} ions (Khan & Martell, 1967b). It has been suggested that the activity change as a function of pH is due to different affinities of metal ions to build complexes with the three forms of ascorbic acid. Besides, also the transformation of oxygen and H_2O_2 during the Fenton reaction is impacted by pH (Kremer, 2003). Oxygen is less reformed from H_2O_2 under acid conditions.

To sum it up, the metal influence on ascorbic acid degradation seems to be fundamentally influenced by the metal type, chelators, pH, the presence of oxygen and their respective interactions. As the effect of metals depends on the availability of oxygen (Boatright, 2016), it might be time limited since oxygen is consumed during oxidation reactions.

3.6 pH

Depending on the used pH, ascorbic acid is in its fully protonated, anionic or double anionic form (Figure 5). It has a pK_a at 4.3 and a second at 11.8 (Bradshaw et al., 2011). The first pK_a is much lower than the second as electrons resulting from deprotonation of the hydroxyl group in position C-3 are resonance stabilized (Matsui et al., 2015).

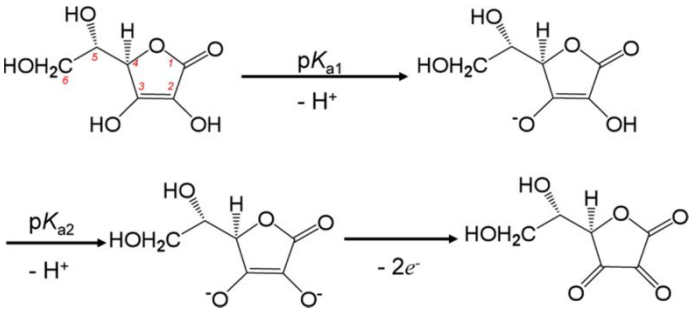


Figure 5: Ascorbic acid as a function of pH.

Taken from Matsui et al. (2015).

At the same pH, the stability of ascorbic acid is influenced by the used acid (Rojas & Gerschenson, 1997). Ascorbic acid degrades faster in a model solution acidified with phosphoric acid than with citric acid. In ethanoic buffer, the oxidation increases with pH (Wilson et al., 1995). The co-presence of sugars or amino acids seems not to influence the tendency. The same trend that is decreasing stability with increasing pH is alike valid for a buffer solution containing protamine (Yamauchi, Nimura, & Kinoshita, 1993), a buffer solution mixed with glucose and lysine and emulsified systems (Gallarate, Carlotti, Trotta, & Bovo, 1999). The emulsified systems have a protective effect in comparison to aqueous solution though. The stability of dehydroascorbic acid follows the same trend as ascorbic acid (Bode, Cunningham, & Rose, 1990). Inter-conversion of ascorbic acid and dehydroascorbic proceeds in acid (pH 4.8 and pH 6.8) but not in alkaline conditions (pH 7.4 and pH 8.1) (Deutsch, 1997a).

3.7 Impact of the food composition

The impact of the co-presence of other food components on ascorbic acid stability has been studied in model solutions. The effect of fructose, xylitol, glucose-mannitol mixture, NaCl and KCl depends on the applied temperature (Rojas & Gerschenson, 2001). In the temperature range 24-45°C, they exhibit a protective effect on ascorbic acid which has been linked to a lower water activity. When temperature is increased to a temperature range of 70-90°C, an inversion of the impact occurs that is ascorbic acid is destabilized by the co-solutes, the most by fructose. The enhanced degradation has been related to an increased non-enzymatic browning.

Steviol glycosides enhance the stability of ascorbic acid and dehydroascorbic acid at ambient temperature which has been assumed to be due the their radical scavenging capacity (Wozniak, Marszalek, & Skvska, 2014).

Polyphenols that are in the oxidized, *ortho*-quinone form are reduced by ascorbic acid which, in turn, is oxidized (Figure 6) (Bradshaw et al., 2011). The stability of green tea polyphenols namely catechins: (-)-epicatechin, (-)-epicatechingallate, (-)-epigallocatechingallate and (-)-epigallocatechingallate (Chen, Zhu, Wong, Zhang, & Chung, 1998) is thus increased. Aka, Courtois, Louarme, Nicolas, and Billaud (2013) have tested the effect of enzymatic oxidation of chlorogenic acid and (-)-epicatechin on the stability of ascorbic acid. Ascorbic acid is destroyed the most in the presence of both substrates and the enzyme polyphenol oxidase. Anthocyanins enhance also ascorbic acid degradation (Shrikhande & Francis, 1974).

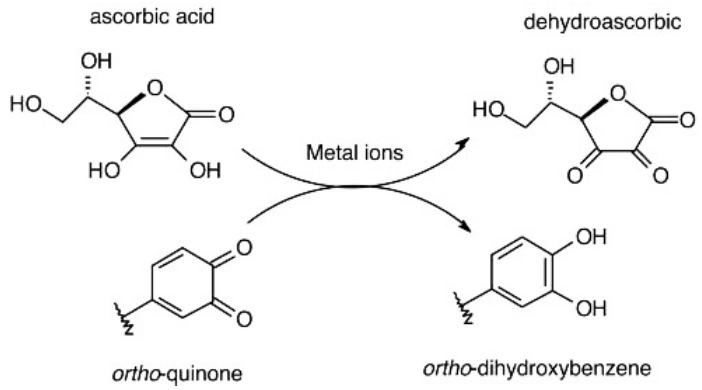


Figure 6: The stability of ascorbic acid decreases in the presence of oxidized polyphenols.

Taken from Bradshaw et al. (2011), adapted version.

The polyphenol subgroup flavonols, in contrast, preserve vitamin C. Ascorbic acid and also anthocyanins are protected by addition of quercetin and quercitrin (Clegg & Morton, 1968). The effect of quercetin is higher than that of quercitrin which has been linked to the additional hydroxyl group.

The stability of ascorbic acid is negatively affected by amino acids during storage (Kacem, Cornell, Marshall, Shireman, & Matthews, 1987). The interaction of proteins with ascorbic acid leads to protein glycation (Slight, Feather, & Ortwerth, 1990). The carbon atom C-1 of ascorbic acid is

incorporated in melanoidins (Davies & Wedzicha, 1994) and also pentosidine (Grandhee & Monnier, 1991). Protein crosslinking in lens extracts occurs only with ascorbic acid but not with sorbitol or glucose when the same molar concentration is used (Ortwerth & Olesen, 1988a). Similarly, non-enzymatic browning in citrus juice is predominantly caused by ascorbic acid compared to sugars (Roig, Bello, Rivera, & Kennedy, 1999). Ascorbic acid does not interfere directly in the Maillard reaction process, but via its degradation products dehydroascorbic acid and 2,3-diketogulonic acid (Reihl, Lederer, & Schwack, 2004; Slight et al., 1990). Glutathione reduces dehydroascorbic acid and prevents hence crosslinking of lens proteins (Ortwerth & Olesen, 1988b).

Also enzymes interact with ascorbic acid but are destroyed by high temperature. Heating of ground broccoli for 15 minutes between 30°C and 60°C provokes transformation of ascorbic acid to dehydroascorbic acid but not in the range 70°C to 90°C which may be due to degradation of ascorbate oxidase (Munyaka, Makule, Oey, Van Loey, & Hendrickx, 2010). Ascorbate oxidase is stable up to 50°C in broccoli but is degraded within 10 minutes at 80°C. The enzyme polyphenol oxidase influences the stability of ascorbic acid, albeit in an indirect way as it enhances polyphenol oxidation (Bradshaw et al., 2011).

When whole food matrices are considered, the stability of ascorbic acid at 37°C increases in the order: water, apple juice, orange juice, blackcurrant drink (Miller & RiceEvans, 1997).

4. Modeling

Mathematical models are often applied to describe time dependent losses. Different modeling approaches exist which depend essentially on the modeling purpose. One main differentiation is the distinction between empiric and mechanistic models. The objective of empiric models is to provide a mathematical model which fits the best to the experimental data which is usually realized by utilizing a high number of parameters (van Boekel, 2009). With rising number of parameters, the fitting increases but simultaneously also the uncertainty of parameter estimates (van Boekel, 2009). A

mechanistic model aims primarily to take into consideration the physical, biological or chemical mechanism. Therefore a limited number of parameters is usually used.

Mechanistic models that are often employed in food chemistry to describe time dependent losses are the zero, first and second order models. These models relate the depletion rate of the molecule of interest (d[A]/dt) to a rate constant (k), which is used for quantitative assessment of the degradation pace. As a function of the curve shape that is linear, exponential or another non-linear, non-exponential behavior, the corresponding model is employed. In the following, differential equations (Table 1: equation 1.1; equation 2.1; equation 3.1) and the linked integrated formulas (Table 1: equation 1.2; equation 2.2; equation 3.2) of the three models are shown, with r corresponding to the reaction rate, [A] to concentration, t to time, $[A]_0$ to the initial concentration and k to the rate constant (van Boekel, 2009).

Table 1: Zero, first and second order models

with corresponding differential equations and integrated formulas.

Model	**Differential equation**		**Integrated formulas**	
Zero order model:	$r = -\frac{d[A]}{dt} = k$	(1.1)	$[A] = -kt + A_0$	(1.2)
First order model:	$r = -\frac{d[A]}{dt} = k\,[A]$	(2.1)	$[A] = A_0 + e^{-kt}$	(2.2)
Second order model:	$r = -\frac{d[A]}{dt} = k\,[A]^2$	(3.1)	$\frac{1}{[A]} = \frac{1}{[A]_0} + kt$	(3.2)

The three differential equations differ in their concentration relationship. The degradation loss expressed as d[A]/dt is not linked to the concentration of A in the zero order model (Table 1, equation 1.1), it is related simply in the first order model (Table 1, equation 2.1) and to the squared concentration of A in the second order model (Table 1, equation 3.1). After integration, this leads to a linear behavior of the concentration of A to the rate constant k in the case of the zero order model (Table 1, equation 1.2), to an exponential one when the first order model is considered (Table 1,

equation 2.2), and to a reciprocal relationship in the case of the second order model (Table 1, equation 3.2).

Models describing the degradation of ascorbic acid are not conclusive (Table 2). Even for one food matrix, orange juice, numerous models were applied in literature (Garcia-Torres et al., 2009). This may have three causal origins. Firstly, the goodness of the model fit and model discrimination is often not carried out but essential as differences between models are often only small (van Boekel, 2008). In many cases, experimental points fit quite well to a zero as well as to a first or second order model. Secondly, degradation must be in a progressed state to carry out model discrimination. A minimum degradation yield of at least 70 % has been proposed by van Boekel (2009). And thirdly, another reason for the ambiguity of degradation behavior of ascorbic acid in literature may be the experimental set-up namely the exposition to oxygen influenced by the geometry, stirring and air tightness of the system.

Most of the time, the first order model has been applied to describe degradation kinetics of ascorbic acid or a variation of it. By applying a first order reversible consecutive reaction model, Serpen and Gökmen (2007) and also Van Bree et al. (2012) obtained beside the rate constant for the oxidation of ascorbic acid to dehydroascorbic acid (k_1), additionally the rate constant for the back reaction from dehydroascorbic acid to ascorbic acid (k_2), and the rate constant for the hydrolysis of dehydroascorbic acid to 2,3-diketogulonic acid (k_3). Serpen and Gökmen (2007) studied thus the influence of Fe^{3+} ions and cysteine, and Van Bree et al. (2012) the impact of headspace oxygen on the three reaction rates. Oey et al. (2006) and also Verbeyst et al. (2013) worked with a biphasic model based on the first order model to incorporate the non-oxidative degradation pathway. Penicaud et al. (2011) integrated the diffusion of ascorbic acid and oxygen in their model. Eisonperchonok and Downes (1982) applied a variation of the second order model that considers instead of the squared ascorbic acid concentration (as shown above in equation 3.1), the concentration of ascorbic acid and of oxygen.

A meta-analysis of rate constants should thoughtfully be carried out as applied models (and thus units), and especially the experimental set-up of most studies differ. The latter influences oxygen

transfers and thus degradation rates (Mohr, 1980). Additionally, the oxygen saturation level of model solutions and food products containing other oxidizable components, can supposed to be different. Also the size of the surface that is exposed to headspace oxygen and stirring of the solution can be assumed to impact the degradation pace. Rate constants are thus only comparable if the same model and the same experimental set-up are applied. In addition, incorporation of the concentration of ascorbic acid as well as of its degradation products in the modeling, which corresponds to a multi-response modeling approach, results usually in more precise degradation rate constants (van Boekel, 2009).

Table 2: Models that were applied in literature to describe degradation paces of ascorbic acid.

Model	Experimental set-up	Matrix	Temperature (°C)	Oxygen	pH	Reference
Second order model	shaken Erlenmeyer flasks	Citrate buffer	30-50	-	6.1	Eisonperchonok and Downes (1982)
First order model	Conical flasks with 100 ml of distilled water	Watercress	82.5; 85; 87.5; 90; 92.5	-	-	Cruz, Vieira, and Silva (2008)
First order model	Sealed pyrex tubes (100 mm length, 16 mm i.d.)	Citrus juice	75; 80; 85; 90; 95; 100	initially: 2.5 mg/L; end: 1.6 mg/L	3.6; 3.7	Dhuique-Mayer et al. (2007)
First order model	Closed Falcon tubes (50 mL)	Sea buckthorn juices	6; 25; 40	-	-	Gutzeit, Baleanu, Winterhalter, and Jerz (2008)
First order model	Tubes	Orange juice serum, Orange juice	70.3-97.6	-	3.6; 3.5	Johnson et al. (1995)
First order model	Tube	Rose hip pulp	70; 80; 90; 95	Anaerobic	3.9	Karhan, Aksu, Tetik, and Turhan (2004)
Zero, first and second order model	Tetra Brik cartons	Orange juice	4; 20; 37; 76; 105	Initially (37°C): 4.45; End (37°C): 2.95	-	Kennedy et al. (1992)
Weibull model	Erlenmeyer flask	Orange juice	20; 25; 30; 35; 40; 45	-	3.7-3.8	Manso et al. (2001)
Biphasic model	Glass vials with rubber septum	Phosphate buffer	100; 110; 120	Initially: 8.11 ppm	7	Oey et al. (2006)
First order model	Hermetically sealed glass flasks without headspace	Humectants, sorbic acid, citric acid	24; 33; 45; 70; 80; 90	Oxygen depletion to 0.2 ppm after 12h (70-90°C)	3.5	Rojas and Gerschenson (2001)
Zero-order model	-	Strawberry juices	8; 28	-	-	Sapei and Hwa (2014)

continued

Table 2–continued

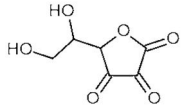
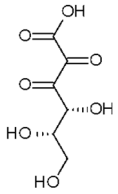
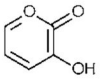
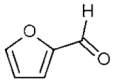
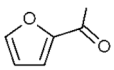
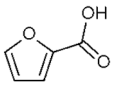
Model	Experimental set-up	Matrix	Temperature (°C)	Oxygen	pH	Reference
First order reversible consecutive reaction model	-	Aqueous solution	90	-	-	Serpen and Gökmen (2007)
Zero order model at low oxygen concentration (≤ 0.63 % O_2), first order irreversible consecutive model, first order consecutive reversible model	500 mL flasks closed with a silicone septum, filled with the juice to gas-to-product ratio (G/P) of 4/1	Model fruit juice, fruit juice	22	Headspace oxygen	3.5	Van Bree et al. (2012)
Biphasic model	Metal tubes, closed, without headspace	Strawberry, raspberry	80; 90; 100; 110; 120	-	-	Verbeyst et al. (2013)
Reversible first order	5 ml of nectar in thermal death time (TDT) tubes (95 mm length, 10 mm i.d), headspace of 2 cm, closed	Nectar of Cupuaçu	60; 70; 75; 80; 90; 99	-	3.2	Vieira, Teixeira, and Silva (2000)
First order model	Sealed glass ampoules, without headspace	Ethanoic buffer	25.15; 22.15; 20.15; 18.15	(In mmol dm^{-3}:) 0.25; 0.24; 0.19; 0.14; 0.13; 0.12; 0.10; 0.09; 0.08; 0.07	1.0; 2.0; 3.0; 3.9; 4.0; 5.0; 6.0; 7.0; 9.5	Wilson et al. (1995)

5. Degradation mechanism

5.1 Detection methods of degradation products

Numerous derivatives of ascorbic acid have been identified by Gas Chromatography (GC-FID) and High Performance Liquid Chromatography (HPLC-UV, HPLC-MS). A quantitative, molar overview is however missing so far due to the number of degradation products and the challenge of their simultaneous detection since their chemical and physical properties differ. Besides, degradation products that are generated depend on treatment conditions. The type and also the quantity vary as a function of the presence of oxygen, temperature, time and pH. At intermediate temperatures, the oxidative pathway dominates the anaerobic degradation pathway (Oey et al., 2006). With increasing temperature dissolved oxygen levels decrease (Penicaud et al., 2012) and simultaneously the importance of anaerobic deterioration increases at temperatures above 100°C. Time determines if intermediates or end products are analyzed and also the pH influences the type and amount of derivatives (Velisek, Davidek, Kubelka, Zelinkova, & Pokorny, 1976; Yuan & Chen, 1998a). Degradation products that have been identified in the past including the constraints of detection methods are reviewed in the next paragraph and are summarized in Table 3. The purpose of the following paragraph is to get an overview of degradation products. It is not complete but aims to highlight main degradation products and detection methods.

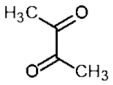
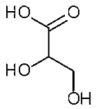
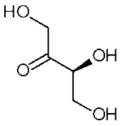
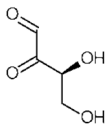
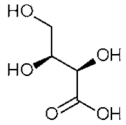
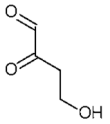
Table 3: Overview of degradation products of ascorbic acid.

Molecule	Degradation condition	Detection method	Author
Dehydroascorbic acid 	Tomatoes	Indirect, Spectrophotometry	Stevens et al. (2006)
	Biological samples	Indirect, HPLC	Lykkesfeldt (2000)
	Fruits and vegetables	LC-MS/MS	Fenoll et al. (2011)
2,3-diketogulonic acid 	Fruits and vegetables	Paired-Ion Reversed Phase Chromatography	Finley and Duang (1981)
	0.2 mol/L DHA, phosphate buffer (0.4 mol/L), pH 7.0, 37°C	^{13}C-NMR	Simpson and Ortwerth (2000)
	Biological samples	OPD trapping, LC-MS/MS	Henning et al. (2004)
3-hydroxy-2-pyrone 	L-dehydroascorbic acid, phosphate buffer, 3h heated under reflux or 25°C, 200 min, pH 2,4,6,8, main degradation product at pH 2 and 4	GC-FID	Velisek et al. (1976) [a]
	100°C, 2 h	HPLC-PDA	Yuan and Chen (1998)
Acetic acid 	L-dehydroascorbic acid, phosphate buffer, 3 h heated under reflux or 25°C, 200 min, pH 2,4,6,8, main degradation product at pH 6 and 8	GC-FID	Velisek et al. (1976)
Furfural 	L-dehydroascorbic acid, phosphate buffer, 3 h heated under reflux or 25°C, 200 min, pH 2,4,6,8	GC-FID	Velisek et al. (1976)
	100°C, 2 h	HPLC-PDA	Yuan and Chen (1998)
2-Acetylfuran 	L-dehydroascorbic acid, phosphate buffer, 3 h heated under reflux or 25°C, 200 min, pH 2,4,6,8	GC-FID	Velisek et al. (1976)
2-furoic acid 	L-dehydroascorbic acid, phosphate buffer, 3 h heated under reflux or 25°C, 200 min, pH 2,4,6,8	GC-FID	Velisek et al. (1976)
	100°C, 2 h	HPLC-PDA	Yuan and Chen (1998)

[a] only main products are presented of this study

continued

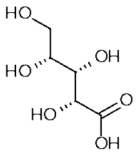
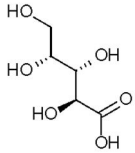
Table 3-continued

Molecule	Degradation condition	Detection method	Author
Glyoxal 	120°C, 120 min, water, pH 3.5	OPD trapping, ESI-tandem MS	Schulz et al. (2007)
Methylglyoxal 	120°C, 120 min, water, pH 3.5	OPD trapping, ESI-tandem MS	Schulz et al. (2007)
Oxalic acid 	30°C or 60°C for 45 min at pH 8, 10	per(trimethylsilylated) GLC-MS	Niemelä (1987)[a]
Diacetyl 	120°C, 120 min, water, pH 3.5	OPD trapping, ESI-tandem MS	Schulz et al. (2007)
Glyceric acid 	30°C or 60°C for 45 min at pH 8, 10	per(trimethylsilylated) GLC-MS	Niemelä (1987)[a]
L-Erythrulose 	0.2 mol/L DHA, phosphate buffer (0.4 mol/L), pH 7.0, 37°C 2.3-DKG, phosphate buffer, 40 h, 23°C, main product	^{13}C-NMR	Simpson and Ortwerth (2000)
L-threosone 	L-dehydroascorbic acid and diketo-L-gulonic acid, phosphate buffer with deuterium dioxide (pH 7.4), room temperature, 7 h, main degradation product	NMR	Nishkawa et al.(2001)
L-threonic acid 	120°C, 120 min, water, pH 3.5 2,3-DKG, H_2O_2, phosphate buffer 2 h	OPD trapping, ESI-tandem MS ^{13}C-NMR	Schulz et al. (2007) Simpson and Ortwerth (2000)
3-deoxy-L-threosone 	120°C, 120 min, water, pH 3.5, main product	OPD trapping, ESI-tandem MS	Schulz et al. (2007)

[a] only main products are presented of this study

continued

Table 3-continued

Molecule	Degradation condition	Detection method	Author
Xylonic acid 	30°C or 60°C for 45 min at pH 8, 10	per(trimethylsilylated)GLC-MS	Niemelä (1987)[a]
Lyxonic acid 	30°C or 60°C for 45 min at pH 8, 10	per(trimethylsilylated)GLC-MS	Niemelä (1987)[a]

Dehydroascorbic acid, the first oxidative degradation product of ascorbic acid, is often analyzed indirectly by reduction of samples solutions with dithiothreitol or tris[2-carboxyethyl]phosphine (TCEP) and subsequent spectrophotometric or HPLC analysis (Lykkesfeldt, 2001; Stevens, Buret, Garchery, Carretero, & Causse, 2006). The amount of dehydroascorbic acid is then calculated by subtracting the reduced amount, often referred to as the total vitamin C amount, by the non-reduced amount. Recently, a method for direct detection of dehydroascorbic acid using LC-MS/MS detection has been established (Fenoll, Martinez, Hellin, & Flores, 2011). The hydrolysis product of dehydroascorbic acid, 2,3-diketogulonic acid, can be detected by Paired-Ion Reversed Phase Chromatography (Finley & Duang, 1981), ^{13}C-NMR analysis (Simpson & Ortwerth, 2000) and by LC-MS/MS detection after *ortho*-phenlyendiamine (OPD) trapping (Henning, Liehr, Girndt, Ulrich, & Glomb, 2014).

Fifteen products, five of them as predominant products namely 3-hydroxy-2-pyrone, 2-furancarboxylic acid, 2-furaldehyde, acetic acid and 2-acetylfuran, were identified by Velisek et al. (1976) using Gas Chromatography. Under these conditions, 3-hydroxy-2-pyrone is the main product at pH 2 and pH 4 and acetic acid at pH 6 and pH 8. Niemela (1987) used trimethylsilyl derivatization to detect inherently non-volatile products. Better stability of derivatives can be achieved by tert-butyldimethylsilyl though (Deutsch, 1997b). For derivatization, a transformation treatment as for example incubation for 60 min at 50-60°C is necessary (Deutsch, 1997b). This is not advantageous if

the effect of temperature is examined as it can be supposed that degradation products due to the treatment are generated. In addition, the derivatization reaction occurs to an incomplete extent impeding accurate quantitation. Another drawback is the injection at high temperature which might also generate new products.

Three of the five main degradation products identified by Velisek et al. (1976) that is furfural, 3-hydroxy-2-pyrone and 2-furoic acid can also be analyzed by HPLC-UV detection (Yuan & Chen, 1998a). Besides another, unidentified molecule which is formed particularly at neutral and higher pHs is detectable by HPLC-UV. A disadvantage of this method consists in the restricted yield of degradation products as UV sensitivity of molecules is a precondition for detection.

Lately, a lot of work has been published using *ortho*-phenylendiamine (OPD) as trapping agent of unstable alpha-dicarbonyls prior to LC-UV or LC-MS analysis. With this technique, especially intermediate products such as dehydroascorbic acid, 2,3-diketogulonic acid and short-chain dicarbonyls can be detected. Schulz et al. (2007) identified thus glyoxal, methylglyoxal, diacetyl, 3-deoxy-L-pentosone, L-threosone and 3-deoxy-L-threosone as degradation derivatives in water (pH 3.5) as well as in baby food treated at 120°C for 120 minutes.

Although more insight in the spectrum of intermediate degradation products of ascorbic acid derivatives could hence be gained, this method has several drawbacks. The trapping agent OPD, accelerates oxidation reactions and leads even to formation of dicarbonyls that are exclusively formed due to the derivatization reaction (Glomb & Tschirnich, 2001). A second disadvantage of this method is the reaction readiness and reaction rate of dicarbonyls with OPD (Henning et al., 2014). A complete derivatization reaction is only completed after 11 h. Short-chain dicarbonyls react faster with OPD than long-chain dicarbonyls and oxalic acid, in turn, does not react at all. Among the dicarbonyls which can be trapped with OPD, dehydroascorbic acid reacts the slowest and moreover via a precursor molecule (Figure 7) which is still detectable to an extent of 10 % after 24 h. Besides that, 7 % of the dehydroascorbic acid-OPD adduct is converted to the 2,3-diketogulonic acid-OPD adduct (Figure 7) during the derivatization leading to inaccurate quantification. Furthermore, given the fact that harsh

conditions entail formation of diasteromeric dicarbonyls-OPD adducts, the pH during derivatization has to be chosen carefully.

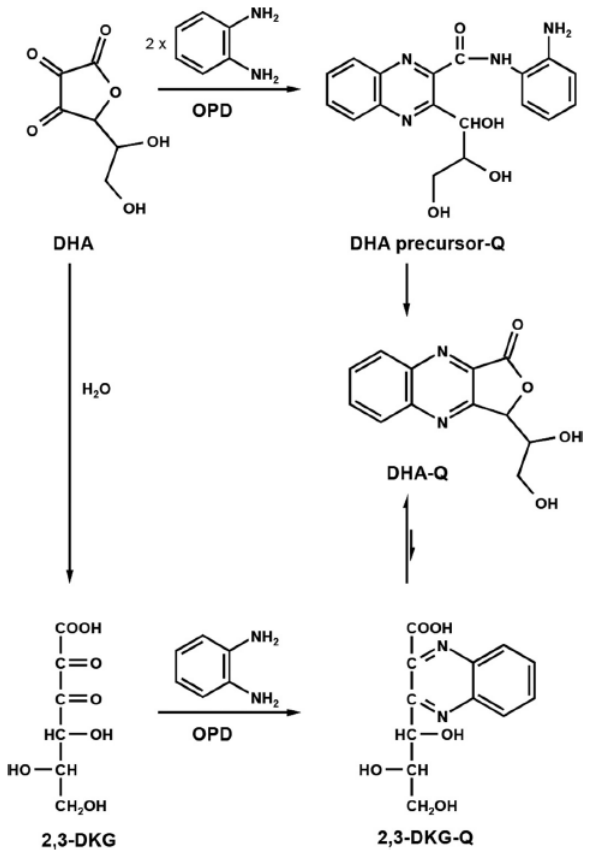


Figure 7: Reaction of *ortho*-phenylendiamine with dehydroascorbic acid.

Taken from Henning et al. (2014).

In real products, the origin of degradation products is difficult to reveal as they can also arise from sugars. Fruit juice concentrates with fortified vitamin C exhibit higher amounts of furfural, 2-furoic acid and 2,5-dimethyl-4-hydroxy-3(2H)-furanone (DMHF) compared to non-fortified ones (Yuan & Chen, 1998b). Louarme and Billaud (2012) used the detection of furfural, 5-(Hydroxymethyl)furfural, 3-hydroxy-2-pyrone and 2-furoic acid to distinguish between thermal and oxidative caused degradation of ascorbic acid during conventional and ohmic heat treatment of fruit products.

5.2 Degradation pathways

Ways to elucidate degradation mechanisms include chemical conclusiveness, isotopic labeling and multi-response modeling. Isotopic labeling of the initial molecule reveals reaction ways with less uncertainty compared to chemical conclusiveness. Multi-response modeling takes into account quantities of all products that are detected over time. With the help of statistics, several reaction pathways can hence be distinguished and reaction rates for each reaction step are provided. However, a high quantitative yield of derivatives that arise from the initial molecule is a precondition for this method (van Boekel, 2009).

The degradation of ascorbic acid entails, due to its sugar related structure, reactions that are typical for sugars transformations namely keto-enol-tautomerism, water elimination, formation of alpha-dicarbonyls and other short C-chain molecules, and ring formations. At intermediate temperatures, the aerobic degradation pathway is predominant (Figure 8) (Oey et al., 2006). The formation of dehydroascorbic acid proceeds via a radical known as monodehydroascorbate radical, semidehydroascorbate or ascorbate free radical (Davey et al., 2000). Dehydroascorbic acid is than hydrolyzed to 2,3-diketogulonic acid. Further reaction cascades entail cleavage of molecules leading to carbon dioxide and short chain molecules (Figure 8). 3-hydroxy-2-pyrone and 2-furoic acid are end-products of the oxidative pathway, furfural of the anaerobic one (Yuan & Chen, 1998a).

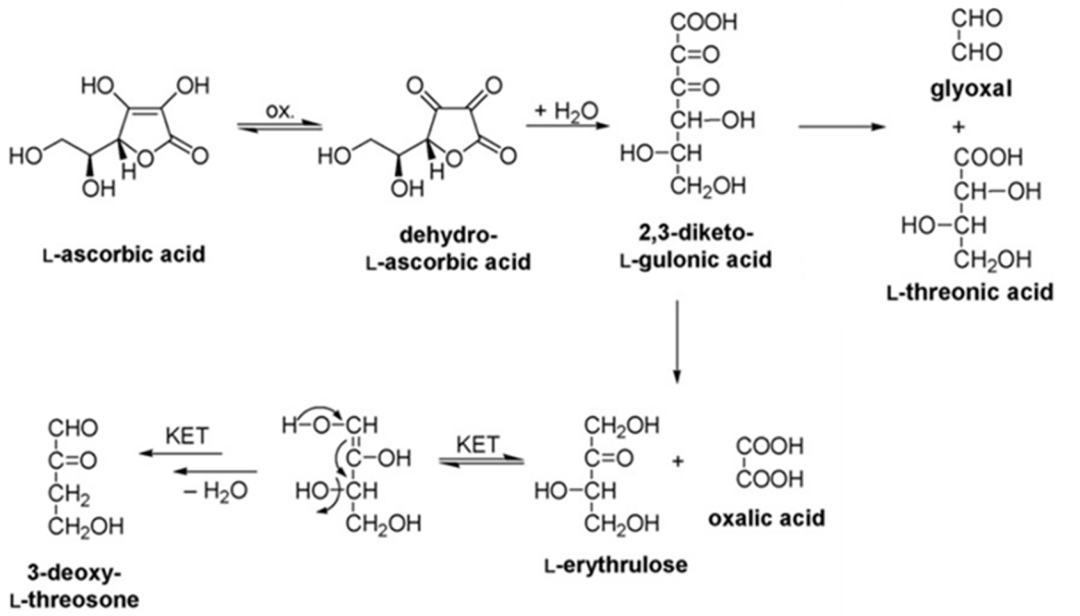


Figure 8: Degradation pathways of ascorbic acid.

Taken from Schulz et al. (2007), adapted version.

Quite a broad spectrum of molecules has been analyzed and degradation pathways could thus be partially elucidated. Further improvement of detection methods is however still needed in order to get a quantitative overview which will help to improve the understanding of the degradation mechanism and also interactions with other molecules. For kinetic studies, the incorporation of dehydroascorbic acid is recommendable, as the bioactive part of vitamin C consists of both molecules. If only ascorbic acid is considered, the nutritional value might be underestimated.

6. Impact of domestic processes

Boiling in water, steaming, microwave heating and stir-frying are common methods to cook food. The term conventional heating is usually referred to food that is heated by conduction which is carried out with or without addition of water. Cooking of food in water leads to diffusion of ascorbic acid (Garrote, Silva, & Bertone, 1988) and thus to an additional loss besides thermal degradation if the water is discarded afterwards. Diffusion losses can be circumvented by steaming. Microwave heating is based on radiation of food products with electromagnetic waves of high frequency. It allows in

comparison to conventional heating faster heating but temperature is inhomogeneously distributed afterwards (Lassen & Ovesen, 1995). Stir-frying, in turn, involves high exposition to air oxygen and high temperature when oil is used. In Table 4, studies that compared the impact of different domestic processes on the loss of vitamin C are listed.

A general recommendation for the method with the highest vitamin C preservation cannot be inferred from literature results. This is probably due to different combinations of impacts namely that of temperature, treatment time, oxygen disposition, kind of food and water addition, which influences the outcome. The majority of studies depicted in Table 4, report that vitamin C is lost the most by conventional boiling. But even for one vegetable, as for example broccoli, the influence of conventional compared to alternative methods is not conclusive. Conventional heating led to the highest loss in the studies Schnepf and Driskell (1994). Zhang and Hamauzu (2004) and Brewer, Begum, and Bozeman (1995), in contrast, reported on equal losses when cooking broccoli conventionally or by microwave. And Vallejo, Tomas-Barberan, and Garcia-Viguera (2002) stated less vitamin C depletion by microwave heating.

When no diffusion loss was possible since food products were not cooked in water, heat degradation of vitamin C in strawberry paste and kiwi purée was higher when they were heated conventionally (loss of 62 % and 27 % respectively) than by microwave heating (loss of 3 % and 1 %) (Benlloch-Tinoco, Igual, Salvador, Rodrigo, & Martínez-Navarrete, 2014; Marszalek, Mitek, & Skapska, 2015). Vallejo et al. (2002) observed the inverse result for orange juice that is higher depletion of vitamin C after a microwave treatment.

Table 4: The impact of different heating methods on the stability of vitamin C.

Food product	Loss trend	Treatment condition	Vitamin C loss	Reference
Strawberry purée	Conventional thermal processing > microwave heating	Conventional: come-up time: 44 min, 90°C, 15 min Microwave: 90°C, 10 s: Microwave: 120°C; 10 s:	62 % 3 % 14 %	Marszalek et al. (2015)
Kiwifruit purée	Conventional thermal processing > microwave heating	Conventional: preheating at 45°C, 18 min, 84°C, 300 s Microwave: 1000 W, 340 s	27 % No significant loss	Benlloch-Tinoco et al. (2014)
Broccoli, Cauliflower, potatoes, corn, peas	Boiled > steamed > Microwave boiled > microwave steamed	Boiling: 1500 mL water (5-8 min) Steaming: 350 mL water (8-30 min) Microwave boiling method: 100 mL water (no water in the case of potatoes) Microwave steaming method: 100 mL water	-	Schnepf and Driskell (1994)
Beans sprouts, green beans, nappa cabbage, spinach	Boiled > stir-fried with water > stir-fried with oil > microwave steaming	Boiling: 1500 mL water, 2-6 min Stir-frying with water: 12 mL water, 3-8 min Stir-frying with oil: 2.5 g coconut oil, 3-9 min) Microwave steaming: 100 mL water, 3-5 min	-	Masrizal, Giraud, and Driskell (1997)
Turnip green and tops	High pressure, conventional boiling > steaming	High pressure: 1500 mL water, 5 min Conventional boiling: 1500 mL, 15 min Steaming: 15 min	100 % (ascorbic acid) 100 % (ascorbic acid) 64 % (ascorbic acid)	Francisco, Velasco, Moreno, Garcia-Viguera, and Elena Cartea (2010)

continued

Table 4-continued

Food product	Loss trend	Treatment condition	Vitamin C loss	Reference
Broccoli	No significant difference between conventional and microwave heating	Conventional: 200 mL, 300 s Microwave: 200 mL boiled water, 600 W, 300 s	Floret: 66 % Stem: 71 %	Zhang and Hamauzu (2004)
Broccoli	No significant difference between boiling, steaming and microwave treatment	Conventional boiling: 1900 mL water, 4 min Steam: 300 mL, 4 min Microwave: 60 mL water, 4 min	20 %	Brewer et al. (1995)
Orange juice	Microwave > conventional heating	Microwave: 1-3 min Conventional heating: 10 min	-	Vikram, Ramesh, and Prapulla (2005)
Broccoli	Microwave > conventional > high pressure > steaming	Microwave: 1000 W, 5 min Conventional: 150 mL water, 5 min High pressure: 150 mL water, 3 min Steaming: 3.5 min	46 % 27 % 25 % 1 %	Vallejo et al. (2002)

Literature Review: 5-methyltetrahydrofolate

Factors that impact the stability of 5-methyltetrahydrofolate

First, general information about folates is given. In connection follows a chapter about factors that impact the stability of 5-methyltetrahydrofolate as this folate vitamer was chosen for supplementation of products. Its degradation mechanism is illustrated as well as the impact of domestic processing. Parts of this chapter were published in the review entitled "Folates in Fruits and Vegetables: Contents, Processing, and Stability" in the journal "Comprehensive Reviews in Food Science and Food Safety". Authors were: Nicolas Delchier, Anna-Lena Herbig, Michael Rychlik, and Catherine M.G.C. Renard.

1. General information

1.1 Physical and chemical characteristics

Folates, also known as vitamin B_9, are a group of vitamers. They have a basic structure in common which can be divided into three parts: a pterin cycle which is connected to *para*-aminobenzoic acid which in turn is linked to glutamic acid (Figure 9). Folate vitamers differ in position N-5 and/or N-10 in which the hydrogen atom is substituted by a methyl, formyl or methylene group. The dominant vitamer in many foods and in humans is 5-methyltetrahydrofolate (Kirsch, Knapp, Herrmann, & Obeid, 2010; Wang, Riedl, & Schwartz, 2013). Folic acid, a synthetic vitamer, is in contrast to natural folates oxidized in position N5-N6 and N7-N8 (Figure 9). When folates are in the poly-glutamate form, the glutamate tail is connected to other glutamates through γ-peptic bonds. Poly-glutamates usually contain up to 8 glutamate residues.

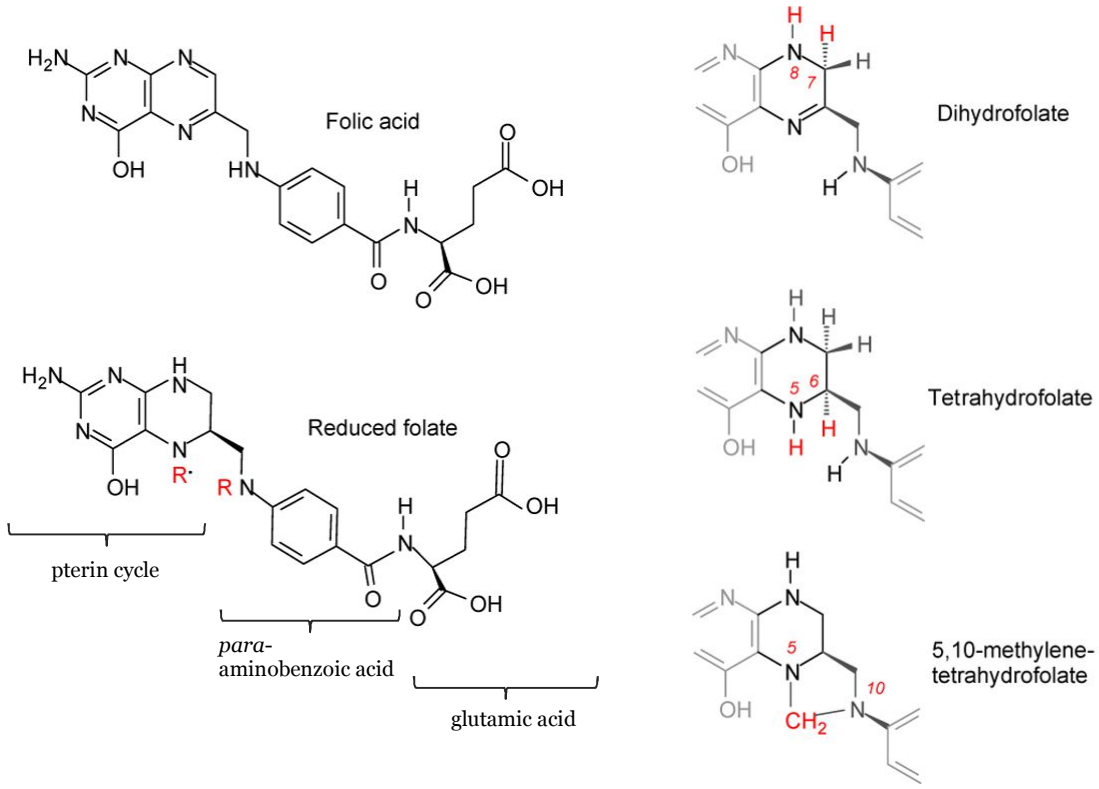


Figure 9: Molecular structure of folic acid and naturally occurring folates.

(R: residue corresponds to hydrogen, a formyl, methyl or a methylene group)

Taken from Strandler, Patring, Jagerstad, and Jastrebova (2015), adapted version.

Folates exhibit several functional groups which are, depending on pH, in a cationic, anionic, zwitterionic or neutral form (Figure 10) (Strandler et al., 2015). In the pH range of fruits and vegetables that is in a range of around pH 3-7, this concerns in particular the α- and γ-carboxyl group of folic acid and in the case of H_4-folate, additionally the form of N_5 (Figure 10). In contrast to H_4-folate, folic acid exists also in an uncharged, neutral form at a pH of around 3.

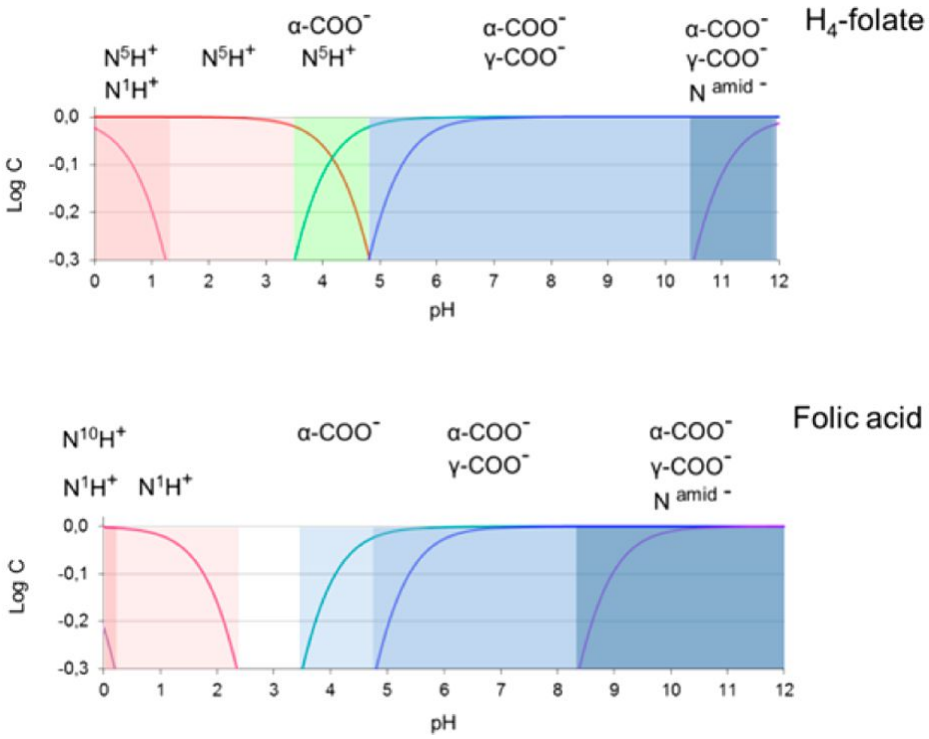


Figure 10: Ionized groups of H_4-folate and folic acid as a function of pH.

pK_a values correspond to the intersection points of the x-axis.

Taken from Strandler et al. (2015), adapted version.

1.2 Bioavailability

As folates are only bioavailable as mono-glutatamates (Halsted, 1989), their deglutamation is important for their nutritional value. The distribution of poly-glutamate lengths is already shifted during processing by the activity of the enzyme γ-glutamyl hydrolase (GGH) (Munyaka, Verlinde, et al., 2010; Wang et al., 2013). Further hydrolysis of poly-glutamates takes place during digestion at the surface of the jejunum (Halsted, 1989). The bioavailability of food folates corresponds to approximately 80 % of that of folic acid (Winkels, Brouwer, Siebelink, Katan, & Verhoef, 2007). Folates are absorbed via the proton-coupled folate transporter PCFT/HCP1 (Qiu et al., 2006). Folate vitamers are converted in the intestine and the liver to 5-methyltetrahydrofolate (Crider, Yang, Berry, & Bailey, 2012). 5-Methyltetrahydrofolate is the form that is primarily absorbed by cells. 5-metyltetrahydrofolate is then converted to tetrahydrofolate which is poly-glutamated again for better cell retention and coenzyme activity (Crider et al., 2012).

To be bioactive, folic acid must be reduced by the enzyme dihydrofolate reductase to dihydrofolate (Figure 11) and then to tetrahydrofolate which is a very slow reaction (Bailey & Ayling, 2009) and thus when high doses (> 200 µg) of folic acid are ingested, also the untransformed form circulates in the serum (Kelly, McPartlin, Goggins, Weir, & Scott, 1997).

1.3 Metabolic role

Folates are a critical cofactor of the one-carbon metabolism which is fundamental for the methylation of RNA, DNA and proteins (Crider et al., 2012; Locasale, 2013). Folate vitamers are reversibly converted in cells (Figure 11). 5-Methyltetrahydrofolate is the vitamer that interacts with the methionine cycle by donating its methyl group to homocysteine which is hereby converted to methionine (Figure 11). Methionine is then transformed in a two-step reaction to *S*-adenosylhomocysteine (SAH). The methyl group is thereby liberated for methylation reactions. Besides, homocysteine is also transformed without the interaction of folates, to cystathionine which is used after further reactions for the anabolism of glutathione and taurine (Figure 11).

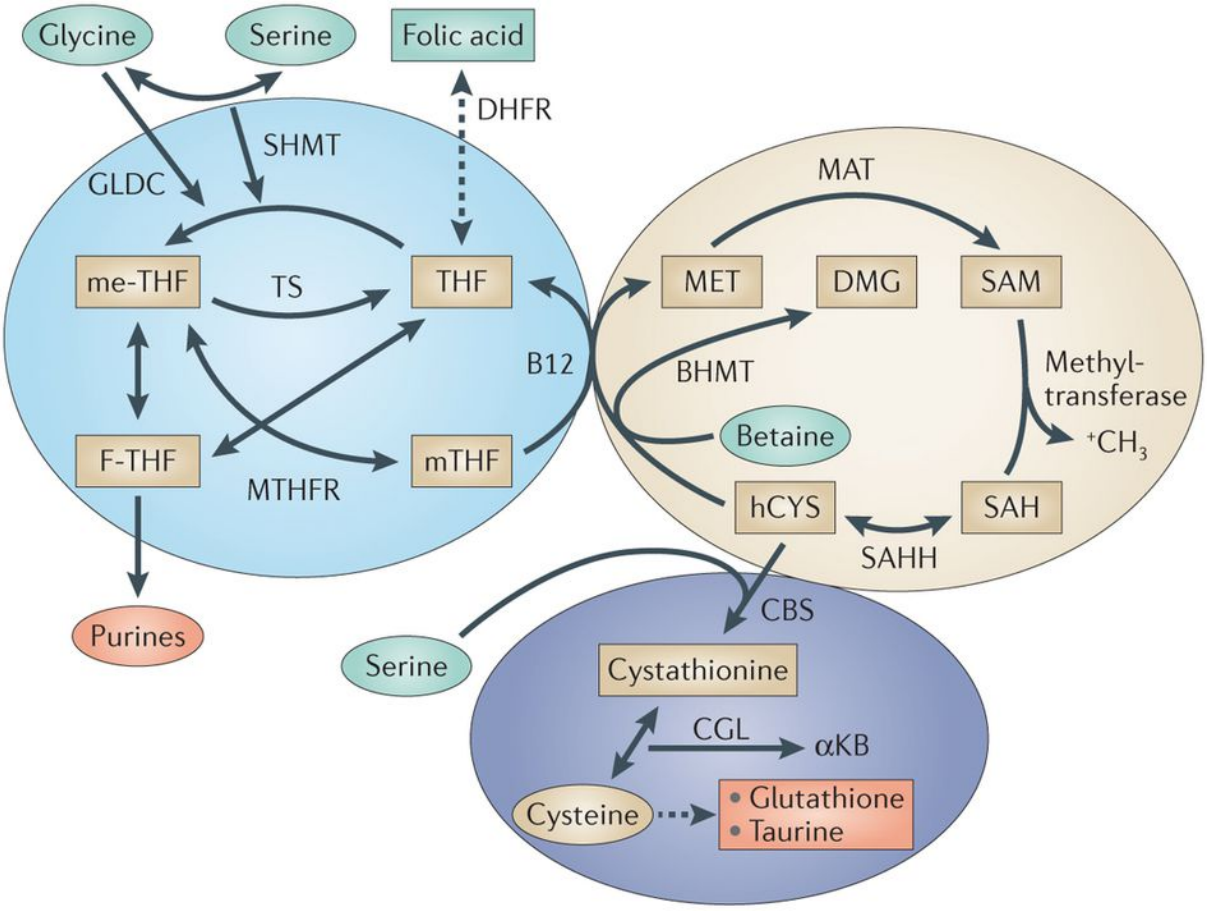


Figure 11: Biochemical role of folates.

The supply of one-carbon units and homocysteine reduction.

Taken from Locasale (2013).

Substrates/Products: Tetrahydrofolate (THF), 5,10-methylene-THF (me-THF), 5-methyltetrahydrofolate (mTHF), 10-formyltetrahydrofolate (F-THF), homocysteine (hCYS), vitamin B_{12} (B12), methionine (MET), *S*-adenosylmethionine (SAM), *S*-adenosylhomocysteine (SAH).

Enzymes: serine hydroxymethyl transferase (SHMT), methylenetetrahydrofolate reductase (MTHFR), methionine adenyltransferase (MAT), *S*-adenosyl homocysteine hydrolase (SAHH), cystathionine synthase (CBS), cystathionine lyase (CGL), α-ketobutyrate (αKB), betaine hydroxymethyltransferase (BHMT), dihydrofolate reductase (DHFR), dimethylglycine (DMG), glycine decarboxylase (GLDC), thymidylate synthase (TS).

1.4 Nutritional impact

Neural tube defects of newborns result from lacking methylation reactions and are thus linked to folate deficiency (Pitkin, 2007). Furthermore, an insufficient intake of folates is assumed to cause a mutagenic reaction that is thymidine substitution by uracil in the DNA sequence (Crider et al., 2012). A sufficient intake of folates, in turn, has been associated with lower homocysteine levels and thus with a protective effect on cardiovascular diseases (Ganguly & Alam, 2015; Rasmussen et al., 2000).

1.5 Requirements

Since food folates are less bioavailable than folic acid, dietary food equivalents (DFE) are usually applied to express recommended intakes. 1 µg DFE has been defined as 1 µg food folate, as 0.6 µg folic acid that is enriched or ingested with food, and as 0.5 µg folic acid that is taken on an empty stomach. An Average Requirement (AR) of 250 µg DFE/day and a Population Reference Intake (PRI) of 330 µg DFE/day has been proposed by the European Food Safety Authority (EFSA). The latter respects a coefficient of variation (CV) of 15 %. In the study of Elmadfa and Freisling (2009), the folate status in 14 European countries was tested and varied between 195-376 µg/day for men and between 194-359 µg/day for women aged between 18 and 64 years. Intakes of people that were 64 years old or older, ranged between 174-311 µg/day for men and between 166-273 µg/day forwomen. Based on the Population Reference Intake proposed by the EFSA, the folate intake of the elderly generation, especially of the female part, is insufficient.

1.6 Supplementation strategies

Enrichment of cereal grain products with folic acid is mandatory in the United States and some other countries and aims to reduce the risk of neural tube birth defects (NTD). The prevalence of NTDs decreased thus by 20-30 % (Pitkin, 2007). In Europe, fortification is allowed and some European countries are contemplating about mandated enrichments (European Food Safety Authority, 2009). So far, folic acid is predominantly used for supplementation. The naturally abundant folate vitamer, 5-methyltetrahydrofolate, has however many benefits. It diminishes for example the potential masking

of a vitamin B_{12} deficiency, does not circulate like folic acid in an unconverted form if high doses are ingested and its bioavailability is not concerned by metabolic anomalies (Scaglione & Panzavolta, 2014). 5-Methyltetrahydrofolate has thus been proposed as fortification alternative (Obeid, Holzgreve, & Pietrzik, 2013; Scaglione & Panzavolta, 2014).

Folic acid is in contrast to 5-methyltetrahydrofolate a fairly stable vitamer. When boiled, it withstands two hours without any destruction (Colman, Green, & Metz, 1975). 5-Methyltetrahydrofolate is more susceptible to deterioration which is a drawback for supplementation but can be circumvented when conditions are well chosen. Factors that influence the stability of 5-methyltetrahydrofolate are going to be reviewed in the following.

2. Stability impacts

2.1 Temperature

5-Methyltetrahydrofolate is susceptible to heat. It degrades at 100°C on a time scale of a few minutes (Chen & Cooper, 1979; Mnkeni & Beveridge, 1983; Paine-Wilson & Chen, 1979). First order models are usually applied to describe the degradation of 5-methyltetrahydrofolate (Chen & Cooper, 1979; Delchier, Ringling, Maingonnat, Rychlik, & Renard, 2014; Indrawati et al., 2004; Liu et al., 2012; Mnkeni & Beveridge, 1983; Nguyen, Indrawati, & Hendrickx, 2003; Oey et al., 2006; Viberg, Jagerstad, Oste, & Sjoholm, 1997). The resulting rate constants at different temperatures are then usually used to determine the activation energy (E_a). Depending on the employed buffer solution and pH, activation energies range from 38 kJ/mol in water to 144 kJ/mol in citric acid-phosphate buffer (pH 3) (Table 5).

Table 5: Stability of 5-methyltetrahydrofolate in different buffer systems.

Medium	pH	Model	Rate constant ($k \times 10^{-3}$ min^{-1})	Activation energy (E_A, kJ/mol)	Reference
Water		First order	$k_{49°C}$ = 4 $k_{65°C}$ = 9 $k_{78°C}$ = 15 $k_{100°C}$ = 32	(46-100°C) 38	Chen and Cooper (1979)
HCl-KCl-buffer	pH 2	First order	$k_{100°C}$ = 70	-	Paine-Wilson and Chen (1979)
HCl-KCl-buffer	pH 3		$k_{100°C}$ = 53	-	
Citrate buffer	pH 3		$k_{100°C}$ = 83	-	
"Universal buffer"	pH 3		$k_{100°C}$ = 254	-	
"Universal buffer"	pH 4		$k_{100°C}$ = 207	-	
"Universal buffer"	pH 5		$k_{100°C}$ = 110	-	
"Universal buffer"	pH 6		$k_{100°C}$ = 103	-	
"Universal buffer"	pH 7		$k_{100°C}$ = 79	-	
				(100°C-130°C)	Mnkeni and Beveridge (1983)
Citrate buffer	pH 3	First order	$k_{100°C}$ = 243	79	
Citrate buffer	pH 4		$k_{100°C}$ = 192	71	
Citrate buffer	pH 5		$k_{100°C}$ = 110	79	
Citrate buffer	pH 6		$k_{100°C}$ = 104	79	
				(60-160°C)	Indrawati et al. (2004)
Citric acid-phosphate	pH 3	First order	$k_{90°C}$ = 125	144	
Citric acid-phosphate	pH 4		$k_{90°C}$ = 115	89	
Citric acid-phosphate	pH 7		$k_{90°C}$ = 14	96	
Acetic acid	pH 3		$k_{90°C}$ = 166	114	
Acetic acid	pH 5		$k_{90°C}$ = 106	81	
Phosphate	pH 7		$k_{90°C}$ = 68	80	

2.2 pH impact

Paine-Wilson and Chen (1979) found that the stability of 5-methyltetrahydrofolate in buffer solution depends markedly on the used buffer ions. At the same pH namely pH 3, 5-methyltetrahydrofolate was the most stable in HCl-KCl buffer and the least stable in "universal buffer" (Table 5). The buffer dependence was confirmed by Indrawati et al. (2004) who observed better stability of 5-methyltetrahydrofolate in citric acid-phosphate buffer than in acetic acid buffer (Table 5). When using the same buffer, 5-methyltetrahydrofolate is more stable at neutral than at acid pH (Indrawati et al., 2004; Ng, Lucock, & Veysey, 2008; Paine-Wilson & Chen, 1979).

However, acidification of broccoli improves the stability of 5-methyltetrahydrofolate. It is stabilized when crushing broccoli with acetate buffer (pH 4.3) instead of water (pH 6.5) (Munyaka, Oey,

Verlinde, Van Loey, & Hendrickx, 2009). Higher preservation of 5-methyltetrahydrofolate in acidified broccoli has been linked to enhanced activity of the enzyme γ-glutamyl hydrolase (GGH) which deconjugates polyglutamates and transforms them into monoglutamates.

2.3 Oxygen

The role of oxygen towards the stability of 5-methyltetrahydrofolate is usually studied by nitrogen flushing of the medium. Dissolved oxygen is hereby substituted by nitrogen which attenuates the degradation of 5-methyltetrahydrofolate (Chen & Cooper, 1979; Mnkeni & Beveridge, 1983; Viberg et al., 1997). However, two points have to be pointed out here.

Firstly, the term "anaerobic condition" should not be used when referred to the above cited studies as oxygen was not removed entirely. In the study of Chen and Cooper (1979), contact of the solution with oxygen could not be avoided as aliquots were regularly withdrawn and thus it can be assumed that oxygen could enter in the system during the heat treatment. Idem, under the experimental set-up of Mnkeni and Beveridge (1983), oxygen was not completely deprived. Although nitrogen was bubbled during 30 minutes through the solution, the dissolved oxygen content was still of 5.3 ppm at room temperature. For comparison, the oxygen saturation level of pure water at 25°C is 8.26 ppm (Penicaud et al., 2012). And also under the conditions of Viberg et al. (1997), the solutions were not completely anaerobic as they compared the effect of an initial oxygen concentration of 0.3 ppm and 6.8 ppm respectively. Indeed, under real anaerobic conditions, that is when the medium and the headspace are completely deprived of oxygen, 5-methyltetrahydrofolate can be heated at 85°C for three hours without any losses (Delchier, Ringling, Cuvelier, et al., 2014).

Secondly, the concentration of dissolved oxygen in the above mentioned studies was only measured at the beginning and not when the temperature of interest was achieved. However, solutions were heated up to 100°C and higher. At these temperatures, oxygen is not soluble anymore (Penicaud et al., 2012). Hence, slower degradation might have rather been due to an oxygen depletion in the headspace and not due to an oxygen decrease in the medium.

5-Methyltetrahydrofolate is a radical scavenger (Gliszczynska-Swiglo, 2007; Joshi, Adhikari, Patro, Chattopadhyay, & Mukherjee, 2001) and thus also sensitive to reactive oxygen species. Hydroxyl radicals cause *N*-dealkylation of amines (Figure 12) via a hydrogen atom transfer (Patro, Adhikari, Mukherjee, & Chattopadhyay, 2005). 5-Methyltetrahydrofolate can thus be assumed to be cleaved in the presence of hydrogen radicals whereby the vitamin activity is finally lost.

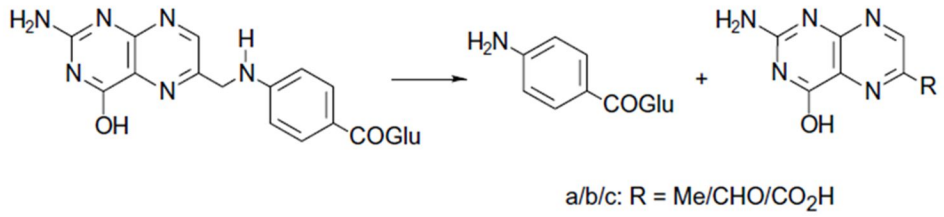


Figure 12: Oxidative *N*-dealkylation of folic acid caused by hydroxyl radicals.

Taken from Patro et al. (2005), adapted version.

2.4 Photo-degradation: the light-oxygen synergy

(published paragraph)

When raw fruits and vegetables are exposed to light, they regulate the content of folates through their metabolism. If they are canned or frozen, they are usually in opaque packaging and thus photo-degradation is not relevant for them either. However, when stored in clear bottles, photo-degradation of fortified folic acid and naturally present folates occurs (Frommherz et al., 2014; Iniesta, Perez-Conesa, Garcia-Alonso, Ros, & Jesus Periago, 2009). Furthermore, photo-degradation is highly relevant during analysis of folates.

Photo-degradation has mostly been studied by exposing folic acid to the fraction of day-light which is rich in energy that is to UV waves. In agreement with thermal treatments, pH influences also the photo-degradation of folates. Scheindlin, Lee, and Griffith (1952) reported better stability of folic acid after exposure to light at pH 6.5 in comparison to pH 4.0. Akhtar, Khan, and Ahmad (2003) corroborated the impact of pH (pH 2-10) on the photo-degradation of folic acid. In agreement with Scheindlin et al. (1952), they observed faster degradation in acid medium.

A synergy between light and the presence of oxygen has been established by several groups. Scheindlin et al. (1952) reported that photo-degradation decreases when oxygen is replaced by nitrogen and Thomas, Suarez, Cabrerizo, Martino, and Capparelli (2000) and Dantola et al. (2010) observed even complete stability of folic acid in the absence of oxygen. Furthermore, Scheindlin et al. (1952) have shown that riboflavin accelerates the photo-degradation of folic acid. Given the fact that light impacts the degradation of folic acid only in the presence of oxygen and that riboflavin has an accelerating effect, it is quite clear that light interacts in the photo-degradation of folates by activating oxygen and not by directly reacting with folates. This hypothesis is reinforced by the fact that triplet oxygen is a diradical and thus can only react with radicals (Choe & Min, 2005).

2.5 Protection by other constituents

Reducing agents counteract the degradation of 5-methyltetrahydrofolate by reconverting the oxidized form in its original form. Many studies address the protective effect of vitamin C. Already by using an equimolar concentration of ascorbic acid, an effective stabilization can be achieved (Rozoy et al., 2012). With rising ascorbic acid concentration, the protective impact increases (Liu et al., 2012; Oey et al., 2006). Oey et al. (2006) related the necessary amount for complete protection of 5-methyltetrahydrofolate to the initial oxygen concentration. They proposed that the concentration of ascorbic acid must be at least as high as the molar concentration of the initial oxygen content. This relationship can however be assumed to be restricted to the used experimental set-up that is a thermal treatment (50-170°C) for 15 minutes in a phosphate buffer (0.1 mol/L) at pH 7, as the effect of ascorbic acid depends not only on the concentration of oxygen but also on pH and time. Ascorbic acid exhibits a greater effect at acid than at neutral pH (Lucock et al., 1995; Ng et al., 2008) and since it is also susceptible to deterioration, the protection is certainly time limited.

Besides ascorbic acid, also epigallocatechin-gallate, catechin, 2-mercaptoethanol and dithiothreitol (DTT) have a protective effect on the stability of 5-methyltetrahydrofolate (Indrawati et al., 2004; Lucock, Green, Hartley, & Levene, 1993; Rozoy et al., 2013). The two latter agents are not relevant for protection in foods but are used to stabilize folates during analyses. 2-Mercaptoethanol is less

effective than ascorbic acid (Obroin, Temperley, Brown, & Scott, 1975) and DTT is active at pH 7.3 and pH 9.0 but not at pH 3.5 (Lucock et al., 1993).

Addition of ascorbic acid to real food products leads also to enhanced thermal stability of 5-methyltetrahydrofolate (Indrawati et al., 2004). Protection of folates in food products was assumed to be related to the antioxidant capacity of the food matrix (Liu et al., 2012).

3. Degradation mechanism

Transformation mechanisms of 5-methyltetrahydrofolate during heat and light treatments and in the presence of sugars are presented in the following. Indicated parts are published in the review of Delchier, Herbig, Rychlik, and Renard (2016).

3.1 Thermal degradation

Verlinde, Oey, Deborggraeve, Hendrickx, and Van Loey (2009) applied multi-response modeling to study the thermal degradation of 5-methyltetrahydrofolate in water (Figure 13). The multi-response modeling was based on the concentrations of 5-methyltetrahydrofolate, 5-methyldihydrofolate, *p*-aminobenzoyl-L-glutamate and the s-triazine derivative 2-amino-8-methyl-4,9-dioxo-7-methyl-p-aminobenzoylglutamate-6,7,8,9-tetrahydro-4*H*-pyrazino(1,2-*a*)-s-triazine. Although they supposed oxidation also via other intermediates (Figure 13, molecules 1.a, 1.b, 1.c, 1.d), they were not included in the modeling. However, the molar yield of detected products was still good indicating that stable, main derivatives were determined. The principal product in heat treated water is the *s*-triazine derivative (Figure 13, molecule 4). Formation of *p*-aminobenzoyl-L-glutamate is enhanced at temperatures above 90°C.

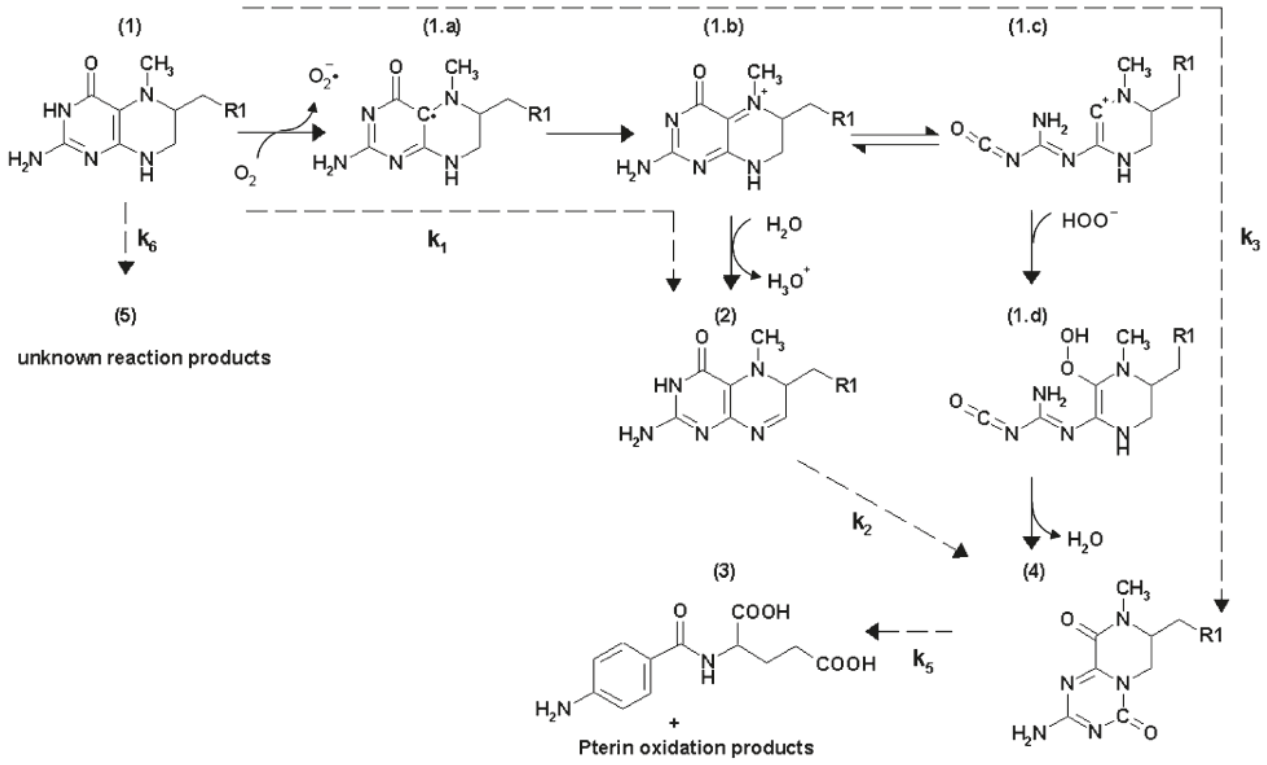


Figure 13: Degradation mechanism of 5-methyltetrahydrofolate.

(*1:* 5-methyltetrahydrofolate, *1.a:* trihydropteridine radical, *1.b:* quinoid 6,7-dihydropteridine, *1.c:* isocyanate, *1.d:* hydroperoxide, *2:* 5-methyldihydrofolate, *3: p*-aminobenzoyl-L-glutamic acid, *4:* 2-amino-8-methyl-4,9-dioxo-7-methyl-p-aminobenzoylglutamate-6,7,8,9-tetrahydro-4*H*-pyrazino(1,2-*a*)-s-triazine))

Taken from Verlinde et al. (2009).

3.2 Photo-degradation

(published paragraph)

Derivatives caused by photo-degradation that were detected in literature are coherent. Akhtar et al. (2003) identified two molecules, 6-carboxy pterine and *p*-aminobenzoyl-L-glutamic acid, and postulated a degradation mechanism. They suggested that folic acid is firstly excited by UV light and subsequently oxidized that is dehydrogenated in position C(9) and N(10), which entails an enamine being more prone to hydrolysis in acid than in alkaline medium. The cleavage of the C(9)-N(10) bond is also known to occur during thermal degradation of folates (Verlinde et al., 2009).

Thomas et al. (2000) determined the same decomposition end-products as Akhtar et al. (2003) and additionally identified the intermediate 6-formylpterine, which is converted to 6-carboxy pterine in the course of the reaction. Off et al. (2005) confirmed the formation of *p*-aminobenzoyl-L-glutamic acid and 6-formylpterine as intermediates. They observed that the latter is converted to 6-carboxy pterine acid after exposure to UV light.

Akhtar, Khan, and Ahmad (1997) examined photo-degradation products in the presence of riboflavin. Besides the derivatives *p*-aminobenzoyl-L-glutamic acid and 6-carboxy pterine, they detected *p*-aminobenzoic acid and pteroic acid.

3.3 Glycation

(published paragraph)

Glycation of the exocyclic amino group of folates in the presence of sugars has been observed in model solution and might also play a role in processed fruits and vegetables. Important work has been carried out to elucidate the molecular mechanism and its influencing factors. Schneider et al. (2002) worked with folic acid and detected the derivative N2-[1-(carboxyethyl)]folic acid (CEF) after a heat treatment at 100°C. Up to 50 % of folic acid was converted to CEF under their conditions. Rychlik and Mayr (2005) quantified CEF in food products by a Stable Isotope Dilution Assay (SIDA). In fortified, baked cookies yields up to 28 % were detected. The extent of CEF formation depended on the used sugar and decreased in the order fructose, glucose, lactose, sucrose.

Verlinde et al. (2010) assessed the stability of 5-methyltetrahydrofolate in the presence of fructose and glucose between 25°C and 90°C. Fructose significantly accelerated degradation of 5-methyltetrahydrofolate, glucose in contrast, did not have an effect. Irrespective of the added fructose concentration namely 1.6 mmol/L and 1.5 mol/L, the rate of degradation remained the same. Addition of ascorbic acid prevented completely the degradation at 100°C during 45 minutes. A degradation mechanism of 5-methyltetrahydrofolate, including the formation of the carboxyethyl derivative has been postulated by Verlinde et al. (2010) by applying multi-response modeling (Figure 14).

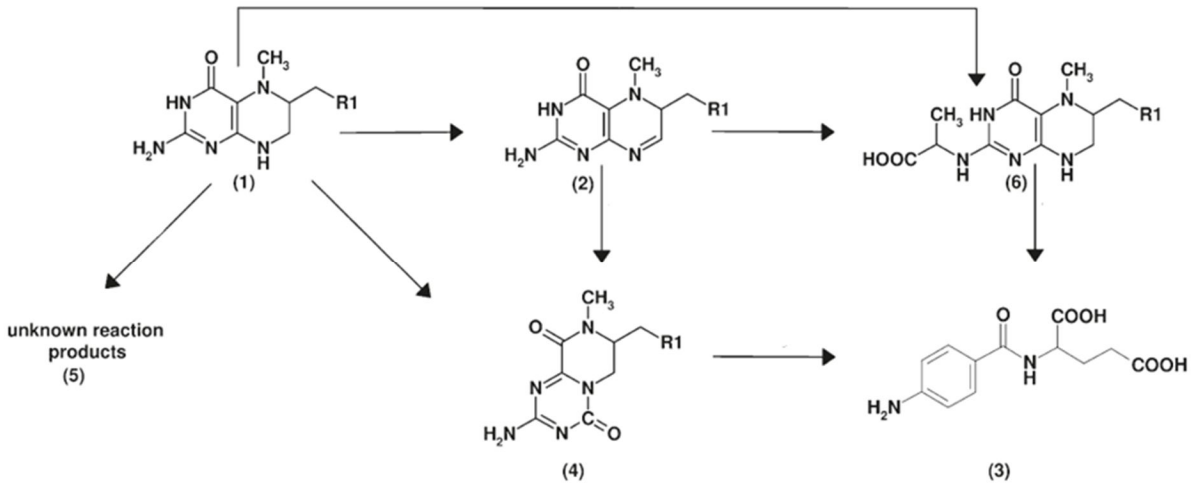


Figure 14: Formation mechanism of N(2α)-[1-(carboxyethyl)]-5-methyltetrahydrofolate (CEF).

(*1:* 5-methyltetrahydrofolate, *2:* 5-methyldihydrofolate, *3: p*-aminobenzoyl-L-glutamic acid, *4:* 2-amino-8-methyl-4,9-dioxo-7-methyl-p-aminobenzoylglutamate-6,7,8,9-tetrahydro-4*H*-pyrazino(1,2-*a*)-s-triazine)

Taken from Verlinde et al. (2010), adapted version.

The importance of glycation of folates in fruit and vegetables remains unclear given the fact that antioxidants are inherently present. In addition, ascorbic acid is often added to processed products, which might entirely prevent glycation. The added amount of ascorbic acid and its own degradation rate might therefore determine whether and when glycation of folates takes place.

4. Influence of domestic heating

Losses of folates during cooking are highly influenced by their water solubility and the linked folate leaching when food is heated in water which is discarded afterwards (Bureau et al., 2015; Dang, Arcot, & Shrestha, 2000; Delchier et al., 2013). However, the degradation extent and mechanism is food type dependent. Folates in spinach are preponderantly lost by diffusion and in green beans mainly by thermally caused deterioration (Delchier, Ringling, Maingonnat, et al., 2014). When the same heating time and vegetable-to-water ratio is used, 5-methyltetrahydrofolate is preserved to a higher extent in Brussel sprouts, cauliflower and broccoli that is to more than 75 %, compared to spinach, savoy cabbage or carrot with a retention percentage between 37-52 % (Holasova, Fiedlerova, & Vavreinova,

2008). Steaming generally increases the vitamin retention (Bureau et al., 2015; Desouza & Eitenmiller, 1986; McKillop et al., 2002). Bureau et al. (2015) found no major losses when different vegetable products were steamed or heated by microwave. Pressure cooking and water boiling, in contrast, had a marked impact. Folates were lost to an extent of 10 % in green beans and to 61 % in minced spinach when cooked under pressure in 400 mL water, and to 26 % and 94 % respectively when boiled in 1 L of water. In the study of Stea, Johansson, Jagerstad, and Frolich (2007), folates in potatoes were not lost when cooked sous-vide, and were preserved to an extent between 59-72 % when boiled (depending on if they were peeled or not) and to 63 % when cooked in oven. When green peas were cooked, similar amounts were retained when different heating methods were applied. Folates were retained to 77 % when boiled, to 75 % when cooked by microwave, to 73 % when steam-cooked and to 71 % when blanched.

When precooked potatoes and green beans were reheated at 100°C between 3 and 15 minutes by a steam-boiler, folates were not lost in the study of Stea et al. (2007). Johansson, Furuhagen, Frolich, and Jagerstad (2008) investigated the impact of different warming-up methods on the folate content of 10 precooked vegetarian ready meals. The studied methods, namely microwave heating (900 W/5.5 minutes), warming-up in a saucepan (until the food reaches 85°C) or heating in an oven (225°C/40 minutes), all caused significant folate losses. A generally, preferable reheating method cannot be inferred from their results as the retention of folates varied a lot. Folates were retained to 63-93 % after stove heating, to 55-89 % after microwaving and to 50-92 % after reheating in an oven.

In general, it seems that losses of folates can be prevented if conditions are well chosen. Boiling is generally not a common reheating method and diffusion losses are thus not relevant for food that is warmed-up. The type of food appears to play a fundamental role as well as the heating method. As outcomes in literature vary quite strongly, it can be concluded that for each food product and each heating method it must be determined separately if the heat treatment causes degradation of folates.

Objectives and approach

General

The European project “OPTIFEL” aims to develop food products to ameliorate the nutritional status of the elderly generation. Proteins, minerals and vitamins are therefore enriched to products on fruit and vegetable basis. The particular objective of the present work consisted in the stability assessment of vitamin C and folates during reheating of food.

Apples and carrots are well appreciated all over Europe and were thus chosen as basis for OPTIFEL products and also as fruit and vegetable example for the present study. They are mainly composed of water (Table 6), contain lots of carbohydrates (principally sugars), iron, antioxidants (apples around 400 mg/100 g of polyphenols, Le Bourvellec et al., 2011, and carrots lots of provitamin A/carotenoids, Table 6) and low to negligible amounts of vitamin C and folates. Sugars and iron are known to accelerate the degradation of vitamin C and folates at intermediate temperatures. The effect of antioxidants depends on their respective redox potential.

Warming-up of food is linked to other constraints than cooking especially in terms of heating temperature and length of time. The recommended minimum temperature during hot keeping of food is almost the same in Europe with 63°C in France (Direction de l'information légale et administrative, 2011) and 65°C in Germany (Bundesinstitut für Risikobewertung, 2008), and aims to prevent growth of spore-forming bacteria. In terms of heating length, a distinction must be made between people’s homes, where food is warmed-up rapidly, and canteen kitchens where products can be kept for several hours at intermediate temperatures. Vitamin C and folates are generally referred to as heat-sensitive molecules and thus it was envisaged in the present work to find conditions under which these two vitamins are effectively preserved and at the same time food safety is ensured.

Table 6: Composition (per 100 g) of raw apple and carrots.

Taken from the Food Composition Databases of the United States Department for Agriculture (USDA, 21.7.2016).

	Unit	Apple	Carrot
Water	g	86	88
Energy	kcal	52	41
Protein	g	0.3	0.9
Total lipid (fat)	g	0.2	0.2
Carbohydrate. by difference	g	13.8	9.6
Fiber. total dietary	g	2.4	2.8
Sugars	g	10.4	4.7
Minerals			
Calcium	mg	6	33
Iron	mg	0.1	0.3
Magnesium	mg	5	12
Phosphorus	mg	11	35
Potassium	mg	107	320
Sodium	mg	1	69
Zinc	mg	0.04	0.24
Vitamins			
Vitamin C, total ascorbic acid	mg	4.6	5.9
Thiamin	mg	0.02	0.07
Riboflavin	mg	0.03	0.06
Niacin	mg	0.09	0.98
Vitamin B6	mg	0.04	0.14
Folate. DFE	µg	3	19
Vitamin B12	µg	0	0
Vitamin A. RAE	µg	3	835
Vitamin E (alpha-tocopherol)	mg	0.18	0.66
Vitamin D (D2+D3)	µg	0	0
Vitamin K (phylloquinone)	µg	2	13

DFE: dietary folate equivalents; RAE: retinol activity equivalents; IU: international unit

Many factors impact the stability of vitamin C in model solution but their respective importance in real food matrices is lacking. It has been found that the availability of oxygen is crucial for the fate of vitamin C and folates but behavior of dissolved oxygen was only assumed and little studied during heat treatments so far. The natural abundant folate vitamer, 5-methyltetrahydrofolate, is less stable than folic acid but can be completely stabilized when conditions are well chosen.

The objective consisted firstly in assessing factors that impact the stability of vitamin C and 5-methyltetrahydrofolate on a laboratory scale (Figure 15). Special focus was set on the study in a real food matrix, an intermediate temperature range and the availability of oxygen. Afterwards, the impact of different reheating methods was aimed to be compared. And finally, with a view to minimize vitamin losses, method parameters were sighted to be optimized by using the gained knowledge of lab-scale experiments.

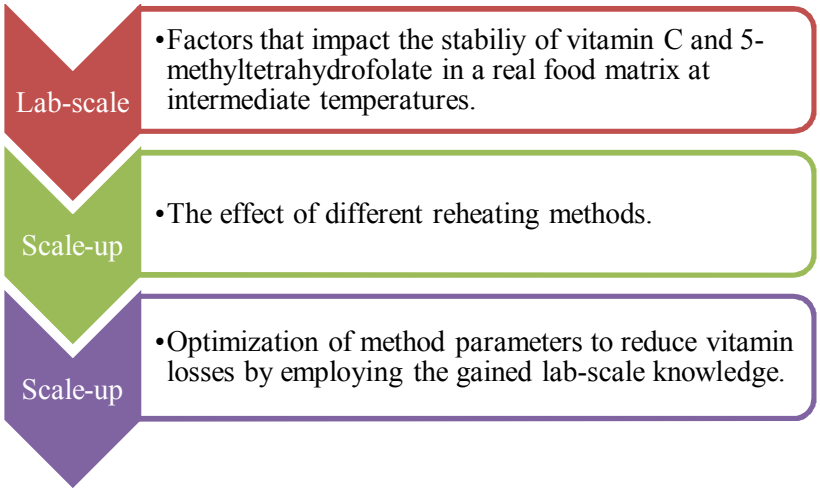


Figure 15: Objectives of the present work.

To fulfill these objectives, the study was divided into four main parts. The first three chapters address the stability of vitamin C and 5-methyltetrahydrofolate on a lab-scale. The last study was dedicated to the scale-up level.

Chapter I

Numerous studies addressed the stability of vitamin C under diverse conditions. The influence of intrinsic factors namely that of metals, polyphenols, sugars, amino acids, proteins, cysteine, enzymes, pH and concentration; and extrinsic factors namely temperature, light and oxygen, has thus been revealed for model solutions. Their respective importance in a real food matrix is lacking though.
In addition, a quantitative comparison of different factors has not been carried out, but is crucial to focus the prevention of vitamin losses on the prevailing degradation impacts(s). Quantitative comparison of impacts by assessing rate constants that were determined in literature is possible only to a limited extent as different models have been applied and thus units of rate constants differ.

Aerobic degradation is always predominant and, at intermediate temperatures in particular, the only pathway that proceeds. Usually, only ascorbic acid is incorporated in the modeling even if the vitamin effect is only lost when dehydroascorbic acid is hydrolyzed to 2,3-diketogulonic acid.

To sum it up, it was hypothesized that stability impacts in food matrices differ from those in model solutions. The aim was to find the quantitatively most important factor in a real food matrix. Therefore, intrinsic and extrinsic factors that modulate vitamin C degradation were compared. One experimental set-up was employed and it was supposed that thus one model can be found that fits well to all time curves. The interest of finding one unique model consisted in getting quantitative, comparable data. The sum of ascorbic acid and dehydroascorbic acid was used for the modeling to avoid underestimation of the vitamin quantity as both are molecules are bioactive.

Chapter II

In the first study, a prevalent impact of the filling height of tubes on vitamin C degradation has been revealed. The influence can be ascribed to different surface-to-volume ratios in tubes and thus to differing oxygen availabilities as other factors remained constant. Hence, the behavior of oxygen at intermediate temperatures was investigated further in the subsequent study.

Aerobic degradation is the only pathway via which vitamin C is lost at intermediate temperatures. Oxygen is soluble up to 100°C. Compared to simple model solutions, food matrices contain numerous other oxidizable compounds which can potentially lead to faster oxygen consumption during heat treatments. When the oxygen content in the system is depleted, vitamin C degradation is slowed down (Van Bree et al., 2012). A dynamic equilibrium exists between headspace and dissolved oxygen. At 20°C and in the presence of vitamin C, oxygen contents during storage of agar gel are higher near the surface as a consequence of faster renewal of dissolved oxygen by oxygen coming from the headspace (Penicaud et al., 2011). As a result, vitamin C is predominantly lost at layers next to the headspace at 20°C. During heat treatments, the content of dissolved oxygen is usually measured only at the beginning and at the end, when the temperature of the medium is cooled down again to room temperature. Oxygen analysis simultaneously to heat treatments is lacking and important to understand profoundly the degradation mechanism of vitamin C at intermediate temperatures.

Under aerobic conditions, Cu^{2+} ions as well as Fe^{3+} ions accelerate the degradation of vitamin C (Boatright, 2016; Serpen & Gökmen, 2007). Even in the presence of Cu^{2+} ions, the stability of ascorbic acid can effectively be increased when oxygen in the system is substituted by argon (Boatright, 2016). It can thus be supposed that also the oxidation of ascorbic acid by Fe^{3+} ions slows down when oxygen is removed. Furthermore, Fe^{3+} ions enhance the oxidation of ascorbic acid to dehydroascorbic acid, as well as the hydrolysis to 2,3-diketogulonic acid. As the accelerating effect of Fe^{3+} ions on the hydrolysis of dehydroascorbic acid might be linked to the production of reactive oxygen species, the effect on this reaction step might also depend on oxygen. When the medium is deprived of oxygen, Fe^{3+} might thus be inactive towards both, the degradation of ascorbic acid and dehydroascorbic acid.

In conclusion, it was hypothesized that the availability of oxygen in model solution differs from that in food matrices and that this will affect the extent of vitamin C degradation as its stability is highly linked to oxygen. Oxygen dynamics in different composed media at 80°C were therefore examined. It was studied in particular if the consumption rate of oxidation reactions dominates and thus leads to rapid oxygen depletion in the medium, or if the pace of oxygen renewal prevails which entails, in contrast, high availability of dissolved oxygen throughout the treatment period. The aim was to know

if oxygen is still available in dissolved form or only in the headspace in the course of time and if it exist an oxygen gradient in the media also at an elevated, intermediate temperature. The concentration effect of ascorbic acid and Fe^{3+} ions, and the influence of temperature was studied. Furthermore, the impact of oxygen on the accelerating effect of Fe^{3+} ions was examined.

Chapter III

The third study addressed the stability of 5-methyltetrahydrofolate. Experiments were carried out at the Chair of Analytical Food Chemistry at the Technical University of Munich (TUM) headed by Professor Rychlik.

Folic acid, a synthetic vitamer, is normally used for supplementation but might mask vitamin B_{12} deficiency. 5-Methyltetrahydrofolate has therefore been proposed as supplementation alternative to cope with insufficient folate intakes (Pietrzik, Bailey, & Shane, 2010b; Scaglione & Panzavolta, 2014). A drawback of this naturally abundant vitamer is however its low stability. 5-Methyltetrahydrofolate is highly susceptible to oxygen and temperature. When the system is completely deprived of oxygen, 5-methyltetrahydrofolate is stable also when heated for 3 h at 85°C (Delchier, Ringling, Cuvelier, et al., 2014). Under aerobic conditions, it is more stable in neutral compared to acid medium (Indrawati et al., 2004; Liu et al., 2012). Fructose accelerates its degradation due to formation of a glycation product (Verlinde et al., 2010).

It can be completely stabilized by ascorbic acid (Oey et al., 2006) which protects 5-methyltetrahydrofolate by reducing its first oxidative product back to the non-oxidized, native form. The amount of ascorbic acid which is necessary to stabilize entirely 5-methyltetrahydrofolate was related to the initial dissolved oxygen concentration by Oey et al. (2006). This relationship can be assumed to be valid only under the specific experimental set-up though. A model solution was used but the relationship in food matrices may be different. Additionally, since ascorbic acid degrades in the course of time, the protection will be time-limited and is counteracted by fructose which accelerates the degradation.

It was hypothesized that 5-methyltetrahydrofolate in a real food matrix can be completely stabilized during reheating. The aim was to find conditions under which 5-methyltetrahydrofolate is not lost. The effect of the food matrix, vitamin C amount and temperature was investigated.

Chapter IV

And finally, the last chapter addressed the impact of different warming-up methods on the stability of vitamin C and 5-methyltetrahydrofolate supplemented to apple and carrot purée. As it has been revealed in chapter III that 5-methyltetrahydrofolate can be effectively preserved in the presence of vitamin C, both vitamins were supplemented together. Production and supplementation of purées was carried out by project partners from the "Centre Technique de la Conservation des Produits Agricoles (CTCPA)" in Avignon/France.

Data about the stability of vitamin C and folates when food is warmed-up is rare. Studies exist which examined the effect of microwave heat treatments. As a function of the heating length, type of food and the addition of water, vitamins are preserved or lost (Benlloch-Tinoco et al., 2014; Bureau et al., 2015; Johansson et al., 2008; Marszalek et al., 2015; Vallejo et al., 2002).

The stability of vitamins when purées are reheated by a microwave and an Actifry ® device, and when held warm in a water bath up to 3 h, was examined. The Actifry ® device was provided by the company "Groupe SEB", also an OPTIFEL partner, who was interested in a new application field. A critical point of the microwave treatment was supposed to be temperature as purées were heated up to ebullition. In the case of the Actifry ® device, stirring and thus high exposition to headspace oxygen was assumed to accelerate vitamin degradation. During the warm-holding of purées in a water bath, the impact of the filling height, which was established in Chapter I on a lab-scale level, was aimed to be verified. In addition, it was assumed that vitamin C degrades at a higher pace near the surface as a consequence of oxygen depletion in the medium and faster oxygen renewal at the top of the medium.

Material and Methods

1. Material

1.1 Chemicals

2-(N-morpholino)-ethanesulfonic acid hydrate, formic acid, 2,2′-Bipyridyl, ascorbic acid, trichloroacetic acid, DL-dithiothreitol, Na_2HPO_4, NaH_2PO_4 x H_20, N-Ethylmaleimide, citric acid monohydrate, Chlorogenic acid, (+)-catechin, (-)-epicatechin, acetic anhydride and toluene-a-thiol were purchased from Sigma-Aldrich (Deisenhofen, Germany) and folic acid from Sigma Aldrich (Steinheim, Germany). 5-Methyltetrahydrofolate was obtained from Schircks (Jona, Switzerland). Acetonitrile, methanol, *ortho*-phosphoric acid (85 %) and iron (III) chloride hexahydrate were provided by VWR (Leuven, Belgium). Ethanol was purchased from Fisher Scientific (Fair Lwan, NJ, USA) and 2,3-Dihydroxybutan-1,4-dithiol from Applichem GmbH (Darmstadt, Germany). [$^{13}C_5$]-5-CH_3-H_4folate, [$^{13}C_5$]-folic acid, disodium hydrogen phosphate, potassium dihydrogen phosphate, sodium acetate trihydrate and acetic acid were obtained from Merck (Darmstadt, Germany) and sodium hydroxide and sodium chloride from J.T. Baker (Deventer, the Netherlands). 4-Coumaric acid and quercetin were purchased from Extrasynthese (Lyon, France) and phloridzin from Fluka (Buchs, Switzerland).

1.2 Food products

Apple purée (brand: POUCE) and carrot purée (brand: HIPP) without added vitamin C, which were used in Chapter I and II, were purchased in a local supermarket in Avignon/France. Apple purée (brand: ALNATURA) and carrot purée (brand: HIPP) without additives, which were taken as supplementation matrix in Chapter III, were obtained in a supermarket in Freising/Germany. Their respective composition, which was indicated on commercial labels, is shown in the supplementary part in Table 12. Apple and carrot purée in Chapter IV were produced by the “Centre Technique de la Conservation des Produits Agricoles (CTCPA)” in Avignon/France.

1.3 Modified food products

1.3.1 Preparation of apple purée serum

Apple purée serum was prepared by the method of Le Bourvellec et al. (2011). Therefore, 50 g of apple purée were weighed in 50 mL corning tubes (Dutcher, Brumath, France) and centrifuged at 13600 g for 10 min. The supernatant was filtrated through a G3 sintered glass filter. The thus obtained liquid was called "apple purée serum".

1.3.2 Supplementation of purées

Supplementation mixtures in Chapter I, II and III were prepared in 50 mL corning tubes with screw caps (Dutcher, Brumath, France). In Chapter I and II, the supplementation matrix (40 g) was weighed in the tubes and ascorbic acid was directly added. For fortifications in Chapter III, folic acid, 5-methyltetrahydrofolate and ascorbic were first pre-solved in water. 10 mL of this solution was then added to 40 g of food product. Enrichments in Chapter IV were carried out by weighing 25 kg of purée and adding 250 mL of a solution that contained 12.625 g ascorbic acid and 50.5 mg 5-methyltetrahydrofolate.

2. Methods

2.1 Thermal treatments

In Chapter I, the supplemented product was transferred to 2 mL tubes (VWR, Leuven, Belgium) which were immersed in a heated water bath. In Chapter II, a double walled vessel (Société Legallais, Montferrier-sur-Lez, France) was used. Temperature of the medium inside the vessel was maintained by circulation of heated water within the two walls. The studied product was pre-heated in a beaker while stirring and then immediately transferred to the double walled recipient. In Chapter III, the studied medium was transferred to 4 mL amber glass vials which were put in a heated silicon oil bath. And in Chapter IV, a domestic microwave of the type Crisp FT439 whirlpool (Whirlpool Corporation, Michigan, USA), an "Actifry ®" device (Groupe SEB, Ecully, France) and a water bath of the type

Royal Catering, RCBM 1/6 150 GN (Royal Catering Corporation, Chemnitz, Germany) were used for the thermal treatment.

After withdrawal, samples were always immediately deep-frozen (≤ -18°C).

2.2 Analytical

2.2.1 Moisture content

Weighing boats were put in a drying cabinet (70°C) for 2 h. After cooling down to room temperature, the vessels were filled with 3 g of sample and stayed again for 4 days at 70°C. The dry matter (%) was determined by dividing the sample weight after drying, by the sample weight before drying, and multiplying by 100. The moisture content (%) was calculated by subtracting the dry matter from 100 %.

2.2.2 Soluble solids (°Brix)

The content of soluble solids was measured with a digital refractometer (PR-101 ATAGO, Norfolk, VA) and expressed in °Brix at 20°C.

2.2.3 Polyphenol analysis

Polyphenols were analyzed as described by Guyot, Marnet, and Drilleau (2001). The method is based on a thioacidolysis reaction for the quantification of procyanidins.

Principle of the method

An incomplete extraction of procyanidins due to interactions with insoluble cell walls (Renard, Baron, Guyot, & Drilleau, 2001) and in addition, an insufficient chromatographic resolution of peaks generated by procyanidins, may lead to an underestimation of the content of procyanidins. To avoid this analytical error, an acid-catalyzed thiolysis was carried out before HPLC analysis. Thereby, procyanidins are depolymerized that is the external units are liberated as monomers and the inner units are transformed to carbocations which react with toluene-α-thiol to thioether adducts. The sample and the media must be dry for the thioacidolysis to prevent incomplete depolymerization (Matthews et al.,

1997). As native monomeric flavan-3-ols cannot be distinguished from those that are formed during the thioacidolysis reaction, the sample has to be analyzed also without thioacidolysis.

Experimental procedure

Thioacidolysis:

After freeze-drying 500 mg of purée, the powder was dissolved in 300 µL of dried and acidified methanol (HCl, 0.4 N). Afterwards, 700 µL of a toluene-α-thiol solution (5 %, v/v, dissolved in dried methanol) was added. The mixture stayed for complete reaction during 30 minutes at 40°C and was subsequently cooled in an ice bath. After filtration through a PTFE-filter (0.45 µm), the solution was analyzed by HPLC-DAD.

Extraction of polyphenols without thioacidolysis:

500 mg of freeze-dried sample were dissolved in 1000 µL dried and acidified methanol (acetic acid, 1 %, v/v). After sonication for 15 minutes, the solution was filtered through a PTFE-filter (0.45 µm) and analyzed by HPLC-DAD.

(+)-catechin, (-)-epicatechin, phloretin xyloglucoside (quantified as phloretin), phloridzin, (-)-epicatechin-benzylthioether (quantified as (-)-epicatechin) were quantified at 280 nm and chlorogenic acid, *p*-coumaroylquinic acid (quantified as *p*-coumaric acid) at 320 nm. Methylated derivatives which were generated during the thioacidolysis reaction were quantified as non-methylated equivalents. The degree of polymerization was calculated as the molar ratio of all flavan-3-ol units to (-)-epicatechin and (+)-catechin that corresponded to end units.

Experimental parameters

Device:	HPLC connected to a Diode-Array Detector (LC-20AD prominence, Shimadzu, Kyoto, Japan)
Pre-column:	LiChrospher PR-18 5 µm column, (Merck, Darmstadt, Germany)
Column:	LiChrospher PR-18 5 µm column (250 mm x 4 mm i.d.) (Merck, Darmstadt, Germany)
Oven temperature:	30°C
Mobile Phase (A):	Acidified water (acetic acid, 2.5 %, v/v)
Mobile Phase (B):	Acetonitrile
Gradient:	Initial condition: 3 % B, linear increase to 9 % B within 5 min, linear increase to 16 % B within 10 min, linear increase to 50 % B within 30 min, linear increase to 90 % B in 3 min, 4 min at 90 % B, linear decrease to 3 % B within 3 min, 5 min at 3 % B
Flow rate:	1 mL/min
Injection volume:	20 µL
Detection wavelengths:	280 nm, 320 nm

2.2.4 Ascorbic acid assay

The method of Stevens et al. (2006) was used for analysis of ascorbic acid and dehydroascorbic acid which is based on a coloration reaction. The latter is performed in micro-plates and absorbance is measured after 50 minutes at 525 nm. Rapid measurement of numerous samples is thus possible. The rate limiting step is the extraction step.

Principle of the method

Coloration reaction:

Ascorbic acid is oxidized by an excess of Fe^{3+} ions to dehydroascorbic acid. The thereby generated Fe^{2+} ions react with 3 mols of 2,2'-Bipyridyl to a colored complex (Figure 16). Absorbance at a wavelength of 525 nm is measured. Quantification is carried out via an external calibration line.

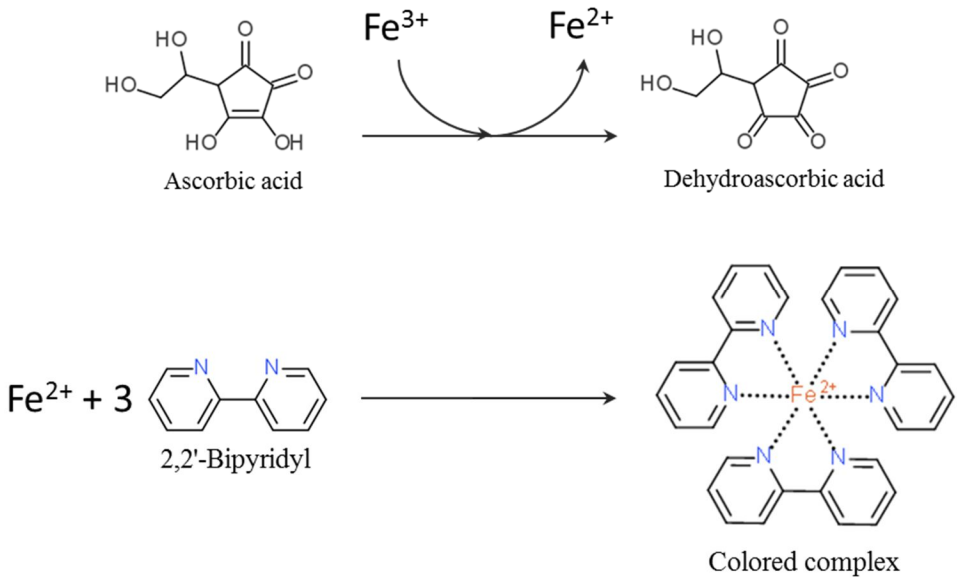


Figure 16: Formation of a colored complex which is used for quantification of ascorbic acid.

Analysis of dehydroascorbic acid and the total vitamin C content:

For the analysis of dehydroascorbic acid, the total vitamin C content is first analyzed which consists of the sum of ascorbic acid and dehydroascorbic acid. Therefore, dehydroascorbic acid is reduced via the reducing agent dithiothreitol (DTT) to ascorbic acid (Figure 17). The content of ascorbic acid is quantified as described above. The amount of dehydroascorbic acid is calculated by subtracting the initial amount of ascorbic acid from the total vitamin C amount.

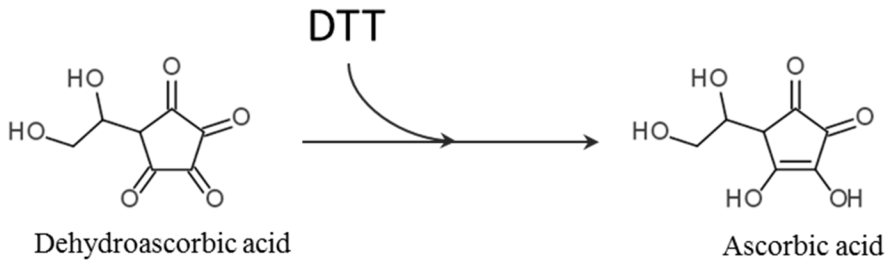


Figure 17: Reduction of dehydroascorbic acid by dithiothreitol (DTT)

to determine the total vitamin C amount.

Experimental procedure

Extraction of ascorbic acid:

An aliquot of 500 mg was weighed in Eppendorf tubes containing already 600 µL of trichloroacetic acid (6%). Tubes were agitated by a vortex and subsequently centrifuged (1K15 model, Bioblock Scientific, Illkirch, France) for 15 minutes at 15500 g. The supernatant was used for analysis of ascorbic acid.

Ascorbic acid analysis:

20 µL of the extract were transferred into a micro-plate well. When the total vitamin C amount was determined, 20 µL of dithiothreitol (DTT, 5 mmol/L) were added. Dithiotreitol was replaced by phosphate buffer (0.4 mol/L, pH 7.4) when the native content of ascorbic acid was analyzed. For complete transformation of dehydroascorbic acid to ascorbic acid, the plate was put in the micro-plate reader where it was agitated for 20 seconds and kept at 37°C for 20 minutes. To stop the reduction reaction, the amount of DTT added in excess, was neutralized by 10 µL N-ethylmaleimide (NEM, 0.5 %). The volume was substituted by 10 µL of phosphate buffer in wells that contained no DTT.

The coloration agent consisted of a mixture of two solutions. Preparation A was composed of 18 mL *ortho*-phosphoric acid (85 %), 31.5 mL ultrapure water, 2.3 g trichloroacetic acid and 0.3 g ferric chloride ($FeCl_3$). Preparation B was a dilution of 2.0 g 2,2-Bipyridyl in 50 mL of ethanol (70 %). 6.6 mL of preparation A and 2.4 mL of preparation B were combined imminently before analysis due to instability of the mixture.

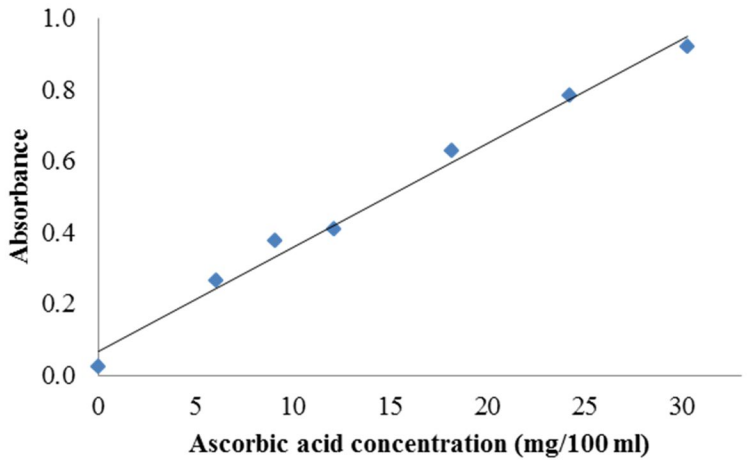
80 µL of the coloration agent was placed in each well to start the coloration reaction. The micro-plate was then placed in the micro-plate reader where it was agitated and kept for complete reaction during 50 minutes at 37°C. Absorbance was measured at a wavelength of 525 nm.

Experimental parameters

Device:	Spectrofluorometer, SAFAS FLX-Xenius
Micro-plate:	96 well micro-plate, Fond F transparent
Absorbance wavelength:	525 nm
Temperature during measurement:	37°C
Measurement time:	50 min
Interval between measurements:	2 min
Bandwidth:	2 nm
Blanc:	Air

Quantification

A calibration line was prepared before each assay in the concentration range 0-30 mg/100 mL. A parent solution containing 100 mg/100 mL of ascorbic acid was therefore prepared. Dilution was carried out with trichloroacetic acid (6 %). A typical calibration line is shown in Figure 18.



Equation of the regression line: $y = 0.029\ x + 0.067$

Coefficient of determination (R^2) = 0.99

Figure 18: Typical calibration line
with corresponding regression equation and coefficient of determination (R^2).

The concentration of ascorbic acid was calculated as follows:

$$\boldsymbol{Concentration\ (mg/100g)} = \frac{\boldsymbol{y-b}}{\boldsymbol{a}}\ \boldsymbol{x}\ \frac{\boldsymbol{0.6+\ h\,x\,m}}{\boldsymbol{m}}$$

y: Absorbance

a: Slope of the regression equation

b: Constant of the regression equation

h: Water content of the food matrix (%)

m: Sample weight (g)

2.2.5 Oxygen availability

Experimental set-up

To study the availability of oxygen during heating of food at intermediate temperatures, a double walled system (Société Legallais, Montferrier-sur-Lez, France) was employed. The set-up permitted maintaining temperature of the medium and oxygen measurement at precise positions from the outside. Oxygen sensor spots (Presens GmbH, Regensburg, Germany) with a diameter of 5 mm were therefore stuck at three positions as indicated in Figure 19. Heated water flew continuously between the double walls to maintain temperature. A cap was stuck with silicon glue on the top to avoid water evaporation. The system was not hermetically closed, oxygen could still enter.

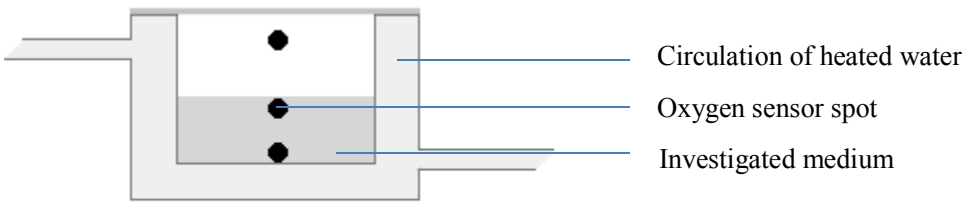


Figure 19: Double walled system containing medium and three oxygen sensor spots.

An advantage of oxygen sensors in comparison to other oxygen quantification methods, as Gaschromatography or Electron Paramagnetic Resonance Spectroscopy (EPR), is the instant measurement (Penicaud et al., 2012). As oxygen is not consumed, analysis by oxygen sensors is a non-destructive method. Non-invasive measurement is possible since the fluorescent material of oxygen sensor spots is excited from the outside of the vessel via an optical fiber. Therefore, the vessel must therefore be transparent. Oxygen sensor spots were preferred to invasive oxygen probes since they permit measurement at exactly the same position in the course of time and also when the medium is changed. Furthermore, bias of oxygen contents due to oxygen renewal when the sensor is inserted or due to temperature changes during the insertion, could thus be excluded

Oxygen sensor spots are conceived for measurement of dissolved and gaseous oxygen. The vessel where sensors are stuck must be transparent and not exceed a thickness of 1 cm. Measurement up to 80°C is possible due to special calibration. Oxygen sensors spots are pre-calibrated by the company. A recalibration must be undertaken after 100.000 measurement points and is done by a two-point calibration. The oxygen content of an oxygen deprived medium and an air saturated environment is used for calibration. As oxygen concentrations depend highly on temperature, a thermometer is connected to the Transmitter during oxygen measurements. The temperature dependence of the luminescence decay and Stern-Volmer constant is known and respected by the device.

Principle of oxygen sensors

The measurement is based on the decrease of a luminescence signal in the presence of molecular oxygen. Therefore, the oxygen spot is equipped with a foil containing the fluorescent material namely polycyclic aromatic hydrocarbons, transition metal complexes of Ru (II), Os (II) and Rh (II), or porphyrins with Pt (II) or Pd (II) as central atom. During the oxygen measurement, light is emitted which excites the fluorescent material and subsequently fluorescence is emitted. When molecular oxygen enters in contact with the luminophoric material (Figure 20), the signal is quenched that is energy is transferred to oxygen and deactivated without radiation. The fluorescence intensity as well as

the decay of the life time of the fluorescent signal can both respectively be used to quantify oxygen via the Stern-Volmer equation.

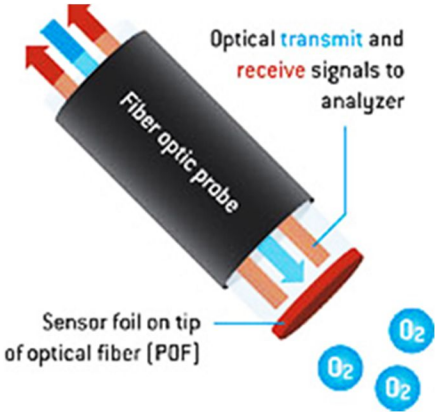


Figure 20: Principle of oxygen sensors.

Taken from Precision Sensing GmbH (PreSens) (http://www.presens.de, 21.7.2016).

Experimental parameters

Company:	Precision Sensing GmbH (PreSens)
Sensor:	Non-Invasive Optical Oxygen Sensors
Transmitter:	Fibox 4
Measurement time:	Instantly
Concentration range:	0-45 mg/L
Limit of detection:	15 ppb
Resolution:	± 0.14 µmol at 2.83 µmol ± 1.4 µmol at 283.1 µmol

Quantification

With rising oxygen concentration, the luminescence intensity as well as the lifetime of the luminescence signal decreases. The relationship is expressed by the Stern-Volmer equation. The indirect proportionality is used to quantify oxygen.

Stern-Volmer equation:

$$\frac{I_0}{I} = \frac{\tau_0}{\tau} = 1 + K_{SV}\ x\ [O_2]$$

I_0: Luminescence intensity without oxygen

I: Luminescence intensity

τ_0: Luminescence decay time without oxygen

τ: Luminescence decay time

K_{SV}: Stern-Volmer constant

$[O_2]$: Oxygen content

2.2.6 Folate measurement by LC-MS/MS detection

The method of Ringling and Rychlik (2013) was applied for quantification of 5-methyltetrahydrofolate and folic acid in Chapter III, which consists of a Stable Isotope Dilution Assay (SIDA) and detection by LC-MS/MS.

Principle of the method

The glutamic acid part of 5-methyltetrahydrofolate and folic acid standards was labeled with five ^{13}C-atoms respectively (Figure 21). As the mass-to-charge-ratio (m/z) of analyte and standard were thus different, two signals were generated by the mass spectrometer. The concentration of the isotopic labeled standard in solution was determined before each assay through the ratio to a non-labeled standard and detection by LC-MS/MS. The concentration of the non-labeled standard, in turn, was analyzed via the ratio to non-labeled folic acid and detection by HPLC-DAD. Stability of 5-methyltetrahydrofolate during analysis was obtained through addition of vitamin C and dithiothreitol.

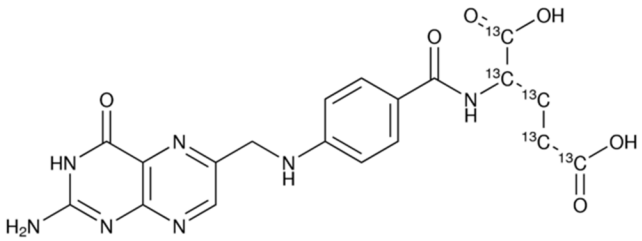


Figure 21: [$^{13}C_5$]-folic acid.

Taken from Sigma-Aldrich (http://www.sigmaaldrich.com, 12.10.2016).

The advantage of isotopic labeled standards in comparison to structurally different ones is that standard and analyte exhibit almost identical chemical and physical properties. Losses during cleaning-up of samples proceed thus to similar extents and the ratio analyte to standard remains equal until the analysis end (Figure 22).

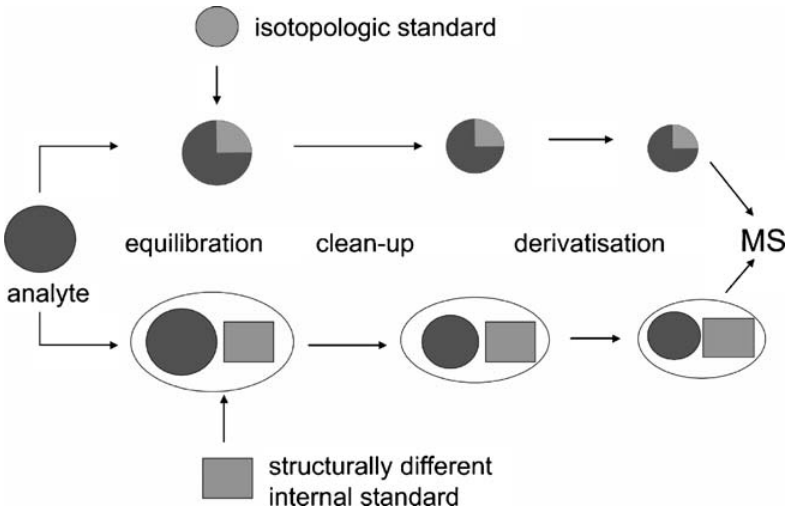


Figure 22: Advantage of an isotopic labeled standard

in comparison to a structurally different, internal standard.

Taken from Rychlik and Asam (2008).

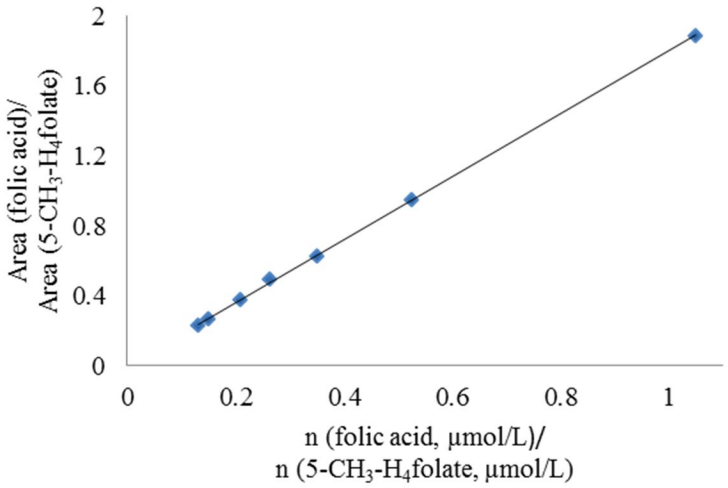
Experimental procedure

Extraction:

A sample aliquot of 0.25 g was weighed in a little Pyrex bottle. The internal standard was added. 10 mL of MES buffer (0.2 mol/L, pH 5) containing 2 % ascorbic acid and 0.1 % dithiothreitol were put into the vessel. The solution was stirred for equilibration during 15 minutes by a magnetic stir bar. The Pyrex bottle maintained 10 minutes in a boiling water bath. Afterwards, the vessel was immediately put in an ice bath until complete cooling to room temperature. The solution was conveyed to a 50 mL centrifugation tube and 10 mL of acetonitrile were added. Tubes were centrifuged at 4°C for 20 minutes at 3000 g. The supernatant was then purified by using a SAX cartridge (500 g, 3 mL, Phenomenex, Torrance, USA). Therefore, the column was first conditioned with two volumes of methanol and two volumes of equilibration buffer which consisted of phosphate buffer (0.01 mol/L, pH 7.0) with 0.02 % dithiothreitol. Afterwards, the sample solution was put on the column and was washed with three volumes of equilibration buffer before running dry. 2 mL elution buffer consisting of 5 % sodium chloride, 0.1 mol/L sodium acetate, 1 % ascorbic acid and 0.1 % dithiothreitol, were used to unfix folates. The solution was passed through a 0.22 µm PVDF membrane filter and measured by LC-MS/MS.

Concentration determination of the standard solution of unlabeled 5-methyltetrahydrofolate:

10 mg of folic acid were pre-dissolved in approximately 20 mL of phosphate buffer (0.1 mol/L, pH 7). The volume of the 100 mL volumetric flask was adjusted with MES buffer. 2 mg of 5-methyltetrahydrofolate were dissolved in 10 mL of MES buffer. 400 µL of the folic acid solution and the 5-methyltetrahydrofolate solution respectively, and 200 mL of MES buffer were pipetted into an amber glass vial. Analysis by HPLC-DAD followed. The concentration of 5-methyltetrahydrofolate was determined via the ratio of folic acid to 5-methyltetrahydrofolate (Figure 23). The following calibration curve was therefore used.



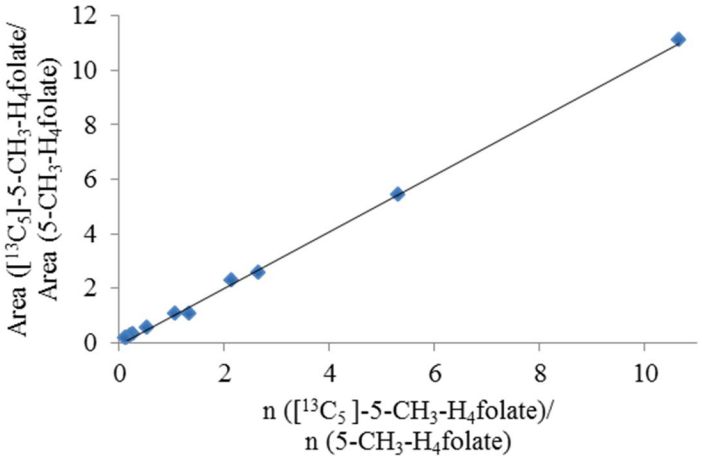
Equation of the regression line: y = 1.7975 x + 0.0009

Coefficient of determination (R^2) = 0.9997

Figure 23: Calibration line to determine the concentration of 5-methyltetrahydrofolate via the ratio to folic acid with corresponding regression equation and coefficient of determination (R^2).

Concentration determination of [$^{13}C_5$]-5-methyltetrahydrofolate and [$^{13}C_5$]-folic acid:

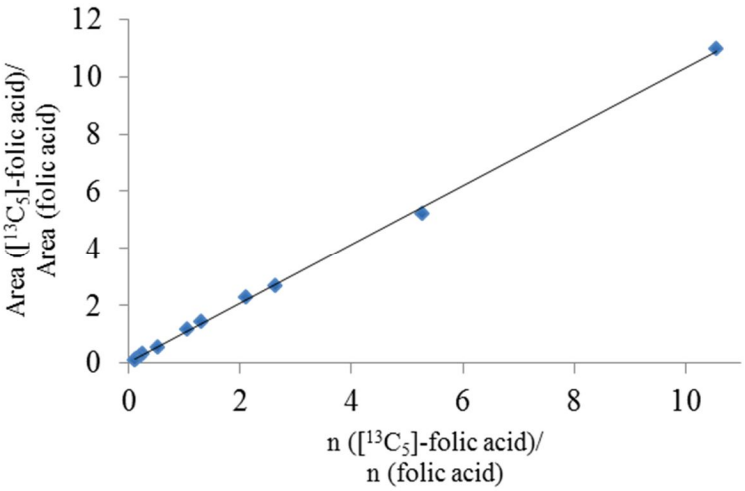
The concentrations of the internal standard solutions, namely that of [$^{13}C_5$]-5-methyltetrahydrofolate and [$^{13}C_5$]-folic acid, were determined before each assay. Therefore, 50 µL of the unlabeled standard solution of 5-methyltetrahydrofolate, prepared as described in the last paragraph and diluted additionally in a ratio 1/20, was added to 100 µL of [$^{13}C_5$]-5-methyltetrahydrofolate standard solution and 1850 µL of elution buffer. Analysis by LC-MS/MS followed. The same was carried for determining the concentration of the labeled folic acid solution. Concentrations of labeled standards were then calculated via the ratio to the known, non-labeled standard concentrations (Figure 24 and Figure 25). The following calibration curves were therefore used:



Equation of the regression line: y = 1.04 x – 0.0835

Coefficient of determination (R^2) = 0.9985

Figure 24: Calibration line to determine the concentration of [$^{13}C_5$]-5-methyltetrahydrofolate with corresponding regression equation and coefficient of determination (R^2).



Equation of the regression line: y = 1.0276 x + 0.0134

Coefficient of determination (R^2) = 0.9992

Figure 25: Calibration line to calculate the concentration of [$^{13}C_5$]-folic acid with corresponding regression equation and coefficient of determination (R^2).

Experimental parameters (HPLC-DAD)

Device:	HPLC connected to a Diode-Array Detector (LC-20A prominence, Shimadzu, Kyoto, Japan)
Pre-column:	C18, 8 x 3 mm (Macherey-Nagel, Düren, Germany)
Column:	Nucleosil reversed phase column, C18, 8 x 3 mm 5 µm, 100 Å (Macherey-Nagel, Düren, Germany)
Oven temperature:	30°C
Mobile Phase (A):	Acetic acid (0.1 %)
Mobile Phase (B):	Methanol
Gradient:	Initial condition: 10 % B, linear increase to 20 % B within 1 min, 20 % B for 1 min, linear increase to 30 % B within 8 min, linear increase to 50 % B within 3 min, linear increase to 100 % B in 2 min, 1 min at 100 % B, linear decrease to 10 % B within 2 min
Flow rate:	0.5 mL/min
Injection volume:	10 µL
Detection wavelength:	290 nm
Retention time (5-CH_3-H_4folate):	18.6 min
Retention time (Folic acid):	22.3 min

Experimental parameters (LC-MS/MS)

Device:	LC-20A prominence HPLC (Shimadzu, Kyoto, Japan), triple quadrupole mass spectrometer (API 4000 Q-Trap, AB Sciex, Foster City, CA, USA)
Pre-column:	C18, 4 x 2 mm (Phenomenex, Torrance, USA)
Column:	HyperClone reversed phase column, C18, BDS, 150 x 3.2 mm, 3 µm, 130 Å (Phenomenex, Torrance, USA)
Oven temperature:	30°C
Injection volume:	10 µL
Flow rate:	0.2 mL/min
Mobile Phase (A):	Formic acid (0.1 %)
Mobile Phase (B):	Acidified acetonitrile (formic acid, 0.1 %)
Gradient:	Initial condition: 2 % B for 2 min, then linear increase to 10 % B within 5 min, 3 min at 10 % B, linear increase to 15 % B within 8 min and then to 100 % in 2 min, 1 min at 100 % B, linear decrease to 2 % B, 2 % B for 9 min
Mass trace ([$^{13}C_5$]-5-CH_3-H_4folate) **Precursor ion (m/z):** **Product ion (m/z):**	 465 313
Mass trace (5-CH_3-H_4folate) **Precursor ion (m/z):** **Product ion (m/z):**	 460 180
Mass trace ([$^{13}C_5$]-Folic acid) **Precursor ion (m/z):** **Product ion (m/z):**	 447 295
Mass trace (Folic acid) **Precursor ion (m/z):** **Product ion (m/z):**	 442 295
Limit of detection (5-CH_3-H_4folate): [a]	0.07 µg/100 g
Limit of detection (Folic acid): [a]	0.10 µg/100 g
Limit of quantification (5-CH_3-H_4folate): [a]	0.22 µg/100 g
Limit of quantification (Folic acid): [a]	0.29 µg/100 g

a: taken from Ringling and Rychlik (2013)

2.2.6 5-methyltetrahydrofolate analysis by HPLC - Fluorescence detection

The method of Ndaw, Bergaentzle, Aoude-Werner, Lahely, and Hasselmann (2001) was applied for quantification of 5-methyltetrahydrofolate in Chapter IV. Main differences compared to the method used in Chapter III are the way of purification, detection and quantification. The two methods are compared at the end of this chapter.

Principle of the method

After extraction, 5-methyltetrahydrofolate is purified via affinity chromatography. Analysis is carried out by HPLC-fluorescence detection. The fluorescence signal is generated by exciting light with a wavelength of 295 nm and the resulting emission wavelength of 356 nm is measured. Quantification is conducted via an external calibration.

Experimental procedure

Extraction:

1 g of sample was weighed in a beaker and 30 mL of phosphate buffer (0.1 mol/L, pH 7) containing 1 % of ascorbic acid, were added. The mixture was homogenized by a spatula and covered by aluminum foil. Recipients were put in a boiling water bath for 10 minutes. After cooling down to room temperature, the mixture was conveyed to a 50 mL volumetric flask and the volume was adjusted with phosphate buffer. The mixture was centrifuged at 4°C for 10 minutes at 5000 g. 10 mL of the supernatant were transferred to a 20 mL volumetric flask. The volume was adjusted with Tris buffer (66 mmol/L, pH 7.8) and the solution was subsequently filtered through a cellulose acetate membrane (80 µm).

Preparation of purification columns:

12 mL of agarose gel (Affigel 10, Biorad, Marnes-la-Coquette, France) were transferred to a glass frit (Pore 3). The supernatant was discarded by a vacuum pump and the gel was washed with a cold solution (4°C) of sodium acetate (0.01 mol/L, pH 4.5). 3 mg of folate binding protein were dissolved in 9 mL of a cold solution (4°C) of sodium carbonate (0.1 mol/L, pH 6.5). The washed agarose gel

was conveyed to the folate binding protein solution and the mixture was stirred at 4°C by a magnetic stir bar for one night. The supernatant was discarded and the gel split into six columns. 5 mL of the sodium carbonate solution were added. After a gelification period of 10 minutes, the columns were washed with 10 mL of sodium carbonate solution and subsequently with 10 mL of phosphate buffer. 5 mL of sodium azide (0.2 %) were run through the columns. When columns were not used, they were stored in the sodium azide solution to prevent growth of microorganisms.

Purification:

The sodium azide stock solution was discarded. After conditioning the column with 5 mL of phosphate buffer, 5 mL of the sample solution were deposited. The column was washed with 10 mL of phosphate buffer and 5 mL of an elution solution (0.02 mol/L dithiothreitol, 0.02 mol/L trifluoroacetic acid) were used to unfix 5-methyltetrahydrofolate. The eluate was collected in a 10 mL volumetric flask containing 40 µL sodium hydroxide solution (60 %) and 200 µL ascorbic acid solution (25 %). The volume was adjusted with elution buffer. 1 mL of the solution was filtered (Millex-AA, 0.45 µm, 25 mm, Millipore, St Quentin en Yvelines, France) and analyzed by HPLC-fluorescence.

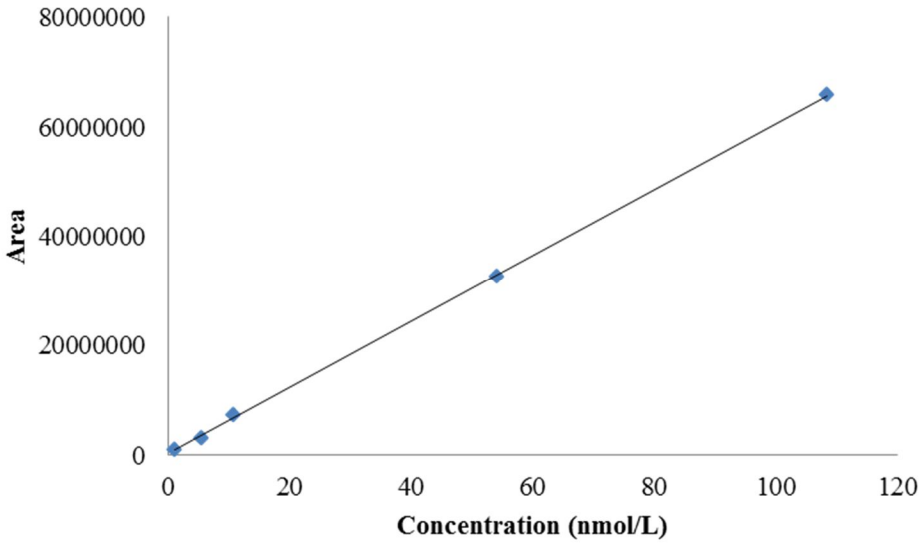
Calculation

The concentration of 5-methyltetrahydrofolate was calculated via a Response Coefficient which was determined as follows. 5 mg of 5-methyltetrahydrofolate were dissolved in 100 mL phosphate buffer containing 1 % ascorbic acid. 100 µL of this solution were diluted again in 100 mL phosphate buffer containing 1 % of ascorbic acid and measured by HPLC-fluorescence. The following equation was used to calculate the Response Coefficient:

$$\text{Response Coefficient} = \frac{\text{Mass x Injection Volume}}{\text{Molar Mass x Final Volume x Dilution x Area}}$$

The Response Coefficient which was used for calculation of the 5-methyltetrahydrofolate content of samples was an average of five independently prepared solutions. Linearity of the fluorescence signal

was tested in the concentration range 1-100 nmol/L (Figure 26). A coefficient of determination (R^2) of 0.9998 was calculated indicating linearity of the signal in the tested concentration range.



Coefficient of determination (R^2) = 0.9998

Figure 26: Detector response to increasing concentrations of 5-methyltetrahydrofolate with corresponding coefficient of determination (R^2).

The concentration of 5-methyltetrahydrofolate in sample solutions was calculated by applying the following equation:

$$\text{Conc.}\left(\frac{\text{mg}}{100\ \text{g}}\right) = \frac{\text{Response Coefficient x Area x Volume x Dilution x Molar Mass x 1000}}{\text{Injected Volume x Mass}}$$

Experimental parameters

Device:	HPLC (Shimadzu, Kyoto, Japan) connected to a fluoroscence detector (RF-10AXL, Shimadzu Inc., Kyoto, Japan)
Pre-column:	LiChrospher RP-18 All Guard (7.5 x 4.6 mm, 5 µm, Altech, France)
Column:	LiChrospher 100RP-18 (250 x 4.5 mm, 5 µm, Altech, France)
Oven temperature:	30°C
Injection volume:	25 µL
Flow rate:	0.8 mL/min
Mobile Phase (A):	Formic acid (0.1 %)
Mobile Phase (B):	Acetonitrile
Gradient:	Initial condition: 5 % B, linear increase to 58 % B within 14 min, linear increase to 100 % B within 3 min, 11 min at 100 % B, decrease to 5 % B within 2 min, 5 min at 5 %
Excitation wavelength:	295 nm
Emission wavelength:	356 nm
Limit of detection (5-CH_3-H_4folate):	0.07 µg/100 g [a]

[a] taken from Ndaw et al. (2001)

2.2.7 Excursus: Comparison of applied folate methods

A detailed overview of folate analysis methods is given in the review of Delchier et al. (2016). The molecule of interest of this work was 5-methyltetrahydrofolate monoglutamate which was supplemented to food purées with negligible natural folate amounts. Detection challenges that are usually encountered when food folates are analyzed, namely the number of folate vitamers that have to be analyzed as well as the glutamate tail that is usually enzymatically hydrolyzed to the monoglutamate form before analysis, did hence not have to be handled. Below, analytical parameters of the employed folate methods are going to be concisely compared.

As folates are usually found in a µg/100 g range in foods, the sensitivity of the method is fundamental for their detection and is provided by both methods. They exhibit both a low limit of detection (Table 7), give linear response in a broad concentration range and show good recovery. One of the main differences is the way of detection which is carried out by a mass spectrometer or a fluorescence detector. Another difference is the purification of extracts which is based on anion-exchange or affinity chromatography. Besides, the calibration for quantification differs. A Stable Isotope Dilution

Assay (SIDA) in the case of the LC-MS/MS method was employed in contrast to an external, one-point calibration in the case of the HPLC-fluorescence method.

A general advantage of the LC-MS/MS over the HPLC-fluorescence method is that different vitamers can be differentiated as they are analyzed individually and not as sum. When only one vitamer is of interest, as it was the case in this work, both are equally suitable methods. The main cost of the LC-MS/MS method, when experimental devices are excluded, is the internal standard and in the case of the HPLC-fluorescence method, the folate binding protein which is used for purification columns.

Table 7: Characteristics of applied folate detection methods.

Method features	LC-MS/MS method	HPLC-fluorescence method
Purification of extracts	Anion-exchange chromatography	Affinity chromatography
Material of purification columns	Quaternary amines	Folate binding proteins
Detection	Triple-quad-MS	Fluorescence detector
Standard	[$^{13}C_5$]-5-methyltetrahydrofolate	5-methyltetrahydrofolate
Calibration	Internal, 11-point-calibration line	External, one-point calibration
Tested concentration range (nmol/L)	0.2-20	1-100
Linearity within the tested concentration range indicated by the coefficient of determination (R^2)	0.9985	0.9998
Limit of detection (µg/100 g)	0.07[a]	0.01[b]
Limit of quantification (µg/100 g)	0.22[a]	N.D.
Recovery (%)	99 (bread) [a]	94 (fruit juice) [b]

a: taken from Ringling and Rychlik (2013); b: taken from Ndaw et al. (2001); N.D.: not determined

3. Different warming-up methods

The impact of three warming-up devices on the stability of vitamin C and 5-methyltetrahydrofolate was studied in Chapter IV. Two devices that are rather encountered at people's homes, namely a microwave (Whirlpool Corporation, Michigan, USA) and an Actifry ® device (Groupe SEB, Ecully, France), and a water bath (Royal Catering Corporation, Chemnitz, Germany) which is usually employed in canteen kitchens to hold food warm, were examined.

3.1 Principle of a microwave oven

When food is heated by a microwave oven, electromagnetic waves are emitted alternately in a positive and negative direction. Dielectric molecules, as water and also other polar molecules, are thus excited and fiction heat is generated (Lassen & Ovesen, 1995; Mathur, 2014). The surface of food products is penetrated by emitted electromagnetic waves. Heat is transferred to the interior by conduction. Microwaves comprise a frequency spectrum from 300 MHz to 300 GHz which corresponds to a wavelength range between 1 m and 1 mm. As microwave radiation is also used in other fields, as telecommunication and military, the range which can be used for industrial, scientific and medical (ISM) has been limited (Hartnett, Irvine, Cho, & Greene, 1999). Microwave ovens usually emit electromagnetic waves with a frequency of 2.5 GHz which corresponds to a wavelength of 12 cm (Jones, Lelyveld, Mavrofidis, Kingman, & Miles, 2002). The power of a microwave oven is expressed in Watt. Microwaves do not ionize molecules or produce radicals. A drawback of this method is that food is inhomogeneously warmed up.

3.2 Warming-up with an Actifry ® device

Actifry ® devices (Figure 27) were developed by the company "Groupe SEB" (Groupe SEB, Ecully, France) to prepare French fries with a low amount of oil. Another, future application field might be the reheating of food. Heating by the Actifry ® device is carried out through heat conduction via

integrated heat plates which is supported by automatic stirring and ventilation of hot air in the headspace.

Figure 27: Principle of heat treatments by Actifry ® devices.

Taken from: http://actifry.tefal.de, 3.9.2016.

3.3 Warm-holding method

The third device was a water bath (Figure 28) of the type RCBM 1/6 150 GN (Royal Catering Corporation, Chemnitz, Germany) equipped with six standardized 1/6 150 GN recipients (width x length x depth: 176 mm x 162 mm x 150 mm) and cover lids supported by silicon gum.

Figure 28: Device which was used to keep purées warm.

Taken from http://images-eu.ssl-images-amazon.com, 3.9.2016.

Water was put in the main vessel up to the restriction line before inserting the six recipients. Warm holding of food was carried out through water evaporation within the main vessel. Temperature was

regulated by the device and verified in the food matrices. Each recipient was used for one respective time point.

4. Model testing and graphical illustration

RStudio (RStudio, Inc., 2009-2015, integrated development environment for R, Version 0.99.483, Boston, MA, retrieved in May, 2015) was used for graphical illustrations, linear and non-linear regression, calculation of Akaike Information Criteria (AIC), plotting residuals and determination of rate constants. The script for testing the fit of a model of zeroth order is shown below as an example. A concise explanation to each command is given and indicated by an arrow and cursive type.

Script for testing the fitting of a model of zeroth order:

```
Apfelmus<-read.csv2(file.choose())
```

➔ *Import data from an Excel sheet saved as csv- file.*

```
Apfelmus
```

➔ *Show the imported data on the Software R interface.*

```
plot(Apfelmus,ylab="",xlab="Time (min)",ylim=c(0,3.0),xlim=c(0,640))
```

➔ *Create a scatterplot in the y-axis range 0 until 3, and the x-axis range 0 until 640.*
➔ *The x-axis is labeled "Time (min)".*

```
mtext(text = "Concentration (mmol/kg)", side = 2, at=3.5,-6.7)
```

➔ *y-axis labeling, above the graph on the left side, at position 3.5/-6.7.*

```
mtext(text = "A", font=2,cex=1.5, side = 4, at=3.5,-1)
```

➔ *Graph labeling "A", above the graph on the right side, at position 3.5/-1,*
➔ *"Font"*= text font, font = 2 corresponds to bold type,
➔ *"cex"*= size of symbols and labels.

```
par(las=1,mar = c(5,5,5,5), mfrow=c(1,1),omi=c(0,0,0,0))
```

➔ *Setting of graphical features,*
➔ *"las": Style of the axis, las=1 corresponds to "always horizontal",*
➔ *"mar": numerical vector defining the form of the graphic by indicating the margin to the outside of the plot in the order bottom, left, top and right,*
➔ *"mfrow": vector indicating the number of rows and the number of columns,*
➔ *"omi": vector defining the margin at the outside in the order bottom, left, top and right in inches.*

```
Apfelmus.zeroorder<-lm(Conc~Time,Apfelmus)

lines(Apfelmus$Time,fitted(Apfelmus.zeroorder))
```

➔ *Plotting linear regression line.*

```
summary(Apfelmus.zeroorder)
```

➔ *Output of regression parameters namely the intercept and constant of the line with respective error estimates and statistical significance that is certainty that values are different from zero.*

```
summary(Apfelmus.zeroorder)$r.squared
```

➔ *Calculation of the coefficient of determination (R^2) indicating the fit of the corresponding regression line to experimental data.*

```
AIC(Apfelmus.zeroorder)
```

➔ *Calculation of the Akaike infomation criterion (AIC),*

```
plot(fitted(Apfelmus.zeroorder),residuals(Apfelmus.zeroorder),
    xlab="Fitted values",ylab="",xlim=c(0,3.0),ylim=c(-0.55,0.55),yaxp=c(-0.5,0.5,10))

mtext(text = "Residuals", side = 2, at=0.73,-1.5)

mtext(text = "B", font=2,cex=1.5, side = 4, at=0.73,-1)

abline(a=0,b=0)
```

➔ *Illustration of residuals.*

Results

Chapter I

The following chapter is an accepted paper which has been submitted to the journal "Food Chemistry". Authors were Anna-Lena Herbig and Catherine M.G.C. Renard. The article is entitled "Factors that impact the stability of vitamin C at intermediate temperatures in a food matrix".

1. Abstract

The study comprises a systematic and quantitative evaluation of potential intrinsic and extrinsic factors that impact vitamin C degradation in a real food matrix. The supernatant of centrifuged apple purée was fortified in vitamin C, and degradation was followed without stirring. Model discrimination indicated better fit for the zero order model than the first order model which was hence chosen for determination of rate constants. pH influenced strongly vitamin C degradation in citrate-phosphate buffer but not in the apple purée serum. To get an idea of the impact of the food matrix, stability in apple purée serum was compared with that in carrot purée. In the latter, stability was slightly higher. Vitamin C degradation rates were not influenced by its initial concentration. The temperature effect was only marked in the temperature range 40-60°C. In the range 60-80°C, filling height of tubes had the greatest impact.

Keywords: Vitamin C degradation, fortification, apple purée serum, dehydroascorbic acid, surface-to-volume ratio

2. Introduction

Vitamin C, consisting of ascorbic acid and dehydroascorbic acid, is an important vitamin in plant foods, and is characterized by its degradability in processing and food preparation. In spite of numerous studies, its degradation is not completely understood. Impact factors are often only known for model solutions (Aka et al., 2013; Kaack & Austed, 1998; Lee & Labuza, 1975; Oey et al., 2006; Rojas & Gerschenson, 1997, 2001; Wilson et al., 1995; Yamauchi et al., 1993) but their importance, especially in a quantitative way, in real food products is lacking.

The predominant pathway of vitamin C degradation in aqueous liquid systems (water activity higher than 0.980) entails oxidation of ascorbic acid to dehydroascorbic acid (Figure 29), which itself promptly degrades to 2,3-diketogulonic acid (Washko, Welch, Dhariwal, Wang, & Levine, 1992).

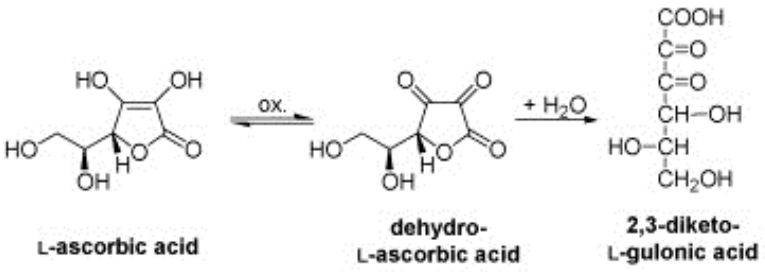


Figure 29: First steps of ascorbic acid degradation via the oxidative pathway.

Taken from Schulz, et al. (2007), adapted version.

By the hydrolysis of dehydroascorbic acid, the molecule looses its vitamin property. With increasing water activity (a_w) or moisture content, the degradation of ascorbic acid increases (Lee & Labuza, 1975). The reaction from ascorbic acid to its oxidized form dehydroascorbic acid and the following hydrolysis to 2,3-diketogulonic acid proceeds in water, without any oxidizers or reducing agents, at the same pace (Serpen & Gökmen, 2007). Fe^{3+} ions accelerate both reaction steps that is oxidation of ascorbic acid and following hydrolysis of dehydroascorbic acid. Cysteine in contrast enhances the reconversion of dehydroascorbic acid to ascorbic acid. During the oxidation of ascorbic acid, oxygen is not incorporated in the molecule itself but serves as acceptor of two electrons. Besides the aerobic degradation pathway via dehydroascorbic acid, ascorbic acid can also be degraded by an anaerobic pathway proceeding by hydrolysis (Schulz et al., 2007; Yuan & Chen, 1998a). The latter is however much slower and occurs only to significant amounts over 120 °C (Dhuique-Mayer et al., 2007; Oey et al., 2006; Verbeyst et al., 2013). Oxygen is therefore an indispensable reaction partner in the intermediate temperature range. When no headspace oxygen is available, degradation of ascorbic acid decelerates after an initial fast depletion of ascorbic acid which can be ascribed to consumption of oxygen as dissolved oxygen contents decrease concomitantly (Robertson & Samaniego, 1986;

Verbeyst et al., 2013). However, changing initial oxygen contents in the range 0.41-3.74 mg/L does not impact the degradation rate of ascorbic acid at 36°C (Robertson & Samaniego, 1986).

The experimental set-up concerning especially the airtightness and stirring of the system in which vitamin C degradation is followed is therefore indispensable to consider. These factors may explain the number of different models applied to describe vitamin C kinetics which range from zero, first and second order models to a biphasic and a Weibull model (Dhuique-Mayer et al., 2007; Eisonperchonok & Downes, 1982; Johnson et al., 1995; Kennedy et al., 1992; Oey et al., 2006; Rojas & Gerschenson, 2001; Sapei & Hwa, 2014; Van den Broeck et al., 1998). In addition, most studies have been carried out only in model solution and behavior in real food products is lacking but indispensable to take into consideration as other components that are naturally present in plant material may interact and stabilize or impair respectively vitamin C stability.

Fructose and glucose enhance vitamin C stability in the temperature rang 24°C to 45 °C and diminish it in the range 70 to 90 °C (Rojas & Gerschenson, 2001). The effect in the storage temperature range has been related to diminished water activity and the one in the processing range to an enhanced non-enzymatic browning.

The impact of polyphenols depends on the kind of polyphenol. Anthocyanidins are described to be protected by vitamin C which acts as inhibitor of the oxidative degradation (Kaack & Austed, 1998). The described protective effect is probably due to reduction of the oxidized form of the polyphenol by ascorbic acid, which is in turn oxidized as has been found for chlorogenic acid and (-)-epicatechin (Aka et al., 2013). Flavonols in turn protect vitamin C (Clegg & Morton, 1968).

Vitamin C stability at 37 °C is higher in blackcurrant, orange juice and apple juice in comparison to water (Miller & RiceEvans, 1997). The effect has been supposed to be a consequence of protection by polyphenols in the fruit juices.

In addition, ascorbic acid oxidation follows a pH tendency in model solutions with higher stability at lower pH (Rojas & Gerschenson, 1997; R. J. Wilson et al., 1995; Yamauchi et al., 1993). The pH dependence of dehydroascorbic acid degradation in phosphate buffer comprising sucrose and EDTA at

23 °C follows the same trend. It degrades significantly faster at pH 7-8 than pH 3-5 (Bode et al., 1990). The impact of concentration was studied by Oey et al. (2006) who stated that vitamin C stability increases with higher concentration and decreases with rising oxygen concentration. The effect of concentration may depend however on the conditions used as the stability of ascorbic acid is related to oxygen. Under the investigated conditions of Oey et al. (2006), a plateau was reached after an initial depletion that may be linked to oxygen consumption in the system. Higher stability was thus probably due to lower oxygen-vitamin C ratio with a constant amount of oxygen when ascorbic acid concentration was high. Dhuique-Mayer et al. (2007) reduced the initial dissolved oxygen content and could thus significantly reduce the degradation rate of ascorbic acid at 90 °C. The effect was however probably not exclusively due to a reduction of dissolved oxygen, as dissolved gases are liberated by heat, but a combination of dissolved oxygen and headspace oxygen depletion. Oxygen depletion in headspace reduces the degradation rate of vitamin C (Van Bree et al., 2012). In addition, an equilibrium between headspace and dissolved oxygen exists that is influenced by oxidations reactions in the food medium. Oxygen mass transfer affects crucially degradation rates under aerobic conditions (Mohr, 1980). Especially for viscous food products where mass transfers are driven by diffusion of molecules, the transfer of oxygen might be limiting for vitamin C degradation.

With temperature increase, dissolved oxygen levels decrease (Penicaud et al., 2012) which counteracts the acceleration of reaction rates with temperature increase described by the Arrhenius equation (Dhuique-Mayer et al., 2007; Manso et al., 2001). Sugars also decrease the oxygen solubility (Joslyn & Supplee, 1949) leading to less oxygen availability for degradation of vitamin C.

The objective of the present study is to systematically investigate potential factors of vitamin C degradation in a quantitative way in order to evaluate their respective significance in apple purée serum. Factors that were studied comprised the intrinsic factors pH, food matrix composition and ascorbic acid concentration and the extrinsic factors temperature and the influence of filling height of experimental tubes. An intermediate temperature range was studied that can be encountered when food is reheated. As the bioactive form of vitamin C includes the oxidized form of ascorbic acid,

dehydroascorbic acid, this concentration was incorporated in the modeling by considering the sum of these two molecules.

3. Material and Methods

3.1 Chemicals

2,2′-Bipyridyl, ascorbic acid, trichloroacetic acid, DL-dithiothreitol, Na_2HPO_4, $NaH_2PO_4xH_20$, N-Ethylmaleimide and citric acid monohydrate were purchased from Sigma-Aldrich (Deisenhofen, Germany). Ortho-phosphoric acid (85%), Iron(III)chloride hexahydrate were obtained from VWR (Leuven, Belgium). Ethanol was provided by Fisher Scientific (Fair Lwan, NJ, USA).

3.2 Supplementation

Apple purée was purchased at a local supermarket and carrot purée was produced by the project partner "Casamas" (Castelltercol, Spain). Both purées contained no added vitamin C. McIlvaine citrate-phosphate buffer at pH of 3.5, 5.5 and 7.5 was prepared for kinetics in buffer solutions. The apple purée serum was obtained by centrifugation of apple purée at 13600 g for 10 min followed by filtration through a G3 sintered glass filter (Le Bourvellec et al., 2011). Supplementation mixtures were prepared in 50 mL corning tubes with screw caps (Dutcher, Brumath, France). Ascorbic acid was added to the respective medium to obtain a concentration of 3 mmol/kg (or 50 mg/100 g), under the study's reference conditions and additionally up to 2 and 5 mmol/kg (40 and 90 mg/100 g) when the impact of concentration was examined. When the pH effect was studied in the apple purée serum, adjustments of pH were carried out with sodium hydroxide (5 mol/L). pH was checked at the end of the thermal treatment and stayed constant. Mixtures were thoroughly vortexed and sonicated for better dissolution of ascorbic acid for approximately one minute. Solutions were transferred to 2 mL micro tubes (VWR, Leuven, Belgium) with caps and filled up to 1.5 mL under standard conditions. Under these conditions, a headspace containing the surrounding air remained. To study the effect of surface-to-volume-ratios, tubes were additionally filled up to 0.5 and 1 mL. Mixtures were immediately frozen

after preparation and defrosted in a water bath before thermal treatment. Freezing and thawing did not affect vitamin C content significantly.

3.3 Thermal treatment

After thawing, tubes were transferred to floating tube racks and immersed in a heated water bath ED-19 from Julabo (Seelbach, Germany). The impact of temperature in the range 40-80°C was studied. The aimed temperature was reached in the tubes after approximately three minutes. The first point of each time curve was excluded from heat treatment and directly refrozen. After withdrawal, tubes were put in an ice bath and immediately frozen (≤ -18 °C). Standard conditions of this study corresponded to heat treatment at 80°C, an added vitamin C concentration of 3 mmol/kg and a filling volume of tubes of 1.5 mL.

3.4 Determination of moisture content

A weighing boat was put in a drying cabinet for 2 h at 70°C. After cooling down to room temperature and weighing, 3 g of sample were put onto it. The vessel stayed for 4 days at 70°C and was weighed again. The dry matter (%) was determined by dividing the sample weight after, by the sample weight before drying, and multiplying by 100. The moisture content (%) was calculated by subtracting the dry matter from 100 %.

3.5 Determination of soluble solids (°Brix)

The content of soluble solids was measured with a digital refractometer (PR-101 ATAGO, Norfolk, VA) and expressed in °Brix at 20°C.

3.6 Polyphenol analysis

Analysis of polyphenols was carried out by Reversed-Phase High Performance Liquid Chromatography (RP-HPLC) – Diode Array Detection (DAD) after thioacidolysis as recently reported by Le Bourvellec et al. (2011).

3.7 Ascorbic acid and dehydroascorbic acid analysis

Ascorbic acid and dehydroascorbic acid quantification was performed as reported by Stevens et al. (2006). Firstly, dehydroascorbic acid is reduced to ascorbic acid by dithiothreitol for determination of the total vitamin C amount that is the bioactive amount consisting of the sum of ascorbic acid and dehydroascorbic acid. Fe^{3+} ions are then added in excess which oxidize ascorbic acid and are reduced in turn to Fe^{2+} ions. The thus generated Fe^{2+} ions react with 2,2'-Bipyridyl to a colored complex. Absorbance was measured at a wavelength of 525 nm on a spectrophotometer (Safas Xenius, Monaco). A calibration line was freshly prepared before each assay.

No vitamin C was detected in the apple purée serum when not supplemented. The non-fortified apple purée serum was then taken as control to verify if coloration or oxidizable molecules generated during heat treatment might lead to a signal increase. The apple purée serum was therefore, as the apple purée serum with added vitamin C, heated at 80°C. A kinetic during 640 minutes was gathered, but no signal increase over time was detected (data not shown).

To avoid bias of modeling as a consequence of differing starting points, the difference in percent of the initial, analyzed vitamin C concentrations of each kinetic curve to the aimed concentration was calculated. The resulting percentage of the difference was used to correct all curve points of the corresponding time curve.

3.8 Kinetic modeling

A zero order model (1) and first order model (2) were fitted to the kinetic of vitamin C determined under the standard condition of this study.

$$c = k_{app} * t + c_0 \qquad (1)$$

$$c = c_0 * e^{-k_{app}t} \qquad (2)$$

c corresponds to the concentration of vitamin C that is the sum of ascorbic acid and dehydroascorbic acid, c_0 to the initial vitamin C concentration, t to the heat treatment duration and k_{app} to the apparent rate constant.

Model discrimination has been conducted through comparison of Akaike information criteria (AIC) indicating the model with the best predictive precision (van Boekel, 2009) and comparison of distribution of residuals to verify homoscedasticity of variance. The functions AIC and residuals provided by Software R were used.

3.9 Software

The software RStudio Team (2015) (RStudio: Integrated Development for R. RStudio, Inc., Boston, MA URL http://www.rstudio.com) has been applied to carry out linear and non-linear regression to experimental data, for determination of AIC values, plotting residuals, for calculation of rate constants, graphical illustration of scattering plots and for model fittings.

4. Results and Discussion

4.1 Matrix simplification

Apple purée is a viscous matrix and needs to be thoroughly mixed to ensure homogeneity. With a view to facilitate sample preparation and especially limit doubts on sample homogeneity, a matrix was searched with similar composition but easier to homogenize. Le Bourvellec et al. (2011) characterized the phenolic and polysaccharidic composition of the liquid supernatant of centrifuged apple purée and observed that the liquid part contains still a considerable amount of polyphenols and low amounts of soluble fibers. With a view to survey if the supernatant of apple purée is suitable for this study; the polyphenol, sugar and moisture content of apple purée and its serum (Table 8) were analyzed as they might potentially enhance or impair respectively vitamin C (Miller & RiceEvans, 1997; Rojas & Gerschenson, 2001). Furthermore initial vitamin C concentrations in apple purée and its serum were determined.

Table 8: Vitamin C, polyphenol (in µmol/kg) and Brix contents (in °) of apple purée and apple purée serum.

(Vit. C: Vitamin C, CAT: (+)-catechin, EC: (-)-epicatechin, PCA: Procyanidins, DPn: average degree of polymerization of flavan-3-ols (catechin+procyanidins), XPL: phloretin-2-xyloglucoside, PLZ: phloridzin, CQA: 5'-caffeoylquinic acid, pCoQA: para-coumaroylquinic acid, TotalFl: total flavonols, TotalP: total polyphenols, s.d.: standard deviation, n.d.: not detectable, a: quantified as phloridzin, b: quantified as p-coumaric acid, c: quantified as quercetin)

	Vit. C	CAT	EC	PCA	DPn	XPL[a]	PLZ	CQA	pCoQA[b]	TotalFl[c]	TotalP	Brix
Apple purée	216	73	254	2044	4	200	686	551	39	86	6943	16
s.d.	*34*	*1*	*3*	*168*	*0.1*	*2*	*3*	*4*	*0.4*	*1*	*55*	*0*
Apple purée serum	n.d.	77	254	976	3	202	691	559	40	71	4809	16
s.d.		*3*	*11*	*22*	*0.02*	*7*	*24*	*20*	*1*	*2*	*9*	*0*

The total phenolic concentration decreased from 6.9 mmol/kg in whole apple purée to 4.8 mmol/kg in the liquid supernatant. The concentration of polyphenols remained thus in the same order of magnitude as that of ascorbic acid planned as reference concentration that is 3 mmol/kg. The polyphenol loss was in accordance to those reported previously for apple serum of twelve different apple varieties (Le Bourvellec et al., 2011). All polyphenol classes were still represented in the supernatant albeit the content of procyanidins and the degree of polymerization of flavan-3-ols decreased. Procyanidins decreased from 2.0 mmol/kg to 9.8 mmol/kg and the degree of polymerization from 3.9 to 2.8. The contents of (+)-catechin, (-)-epicatechin, phloretin-2-xyloglucoside, phloridzin, 5'-caffeolyquinic acid, para-coumaroylquinic acid and total flavanols did not decrease at all. Concerning the sugar content, the same °Brix was found in the liquid part (16.1 ± 0.1 °Brix) as in the whole apple purée (15.9 ± 0.4 °Brix), and thus the potential impact of sugars was considered to be the same in both matrices. The water content in both apple matrices was high but not different (serum: 80.1 ± 0.9; apple purée: 78.0 ± 1.6). Fernández-Salguero, Gómez, and Carmona (1993) determined a moisture content of apples of 88.5 ± 0.3 % and a corresponding water activity (a_w) of 0.988 ± 0.002.

The concentration of vitamin C before supplementation was 0.2 mmol/kg in apple purée, under the limit of detection in the apple purée serum and thus negligible compared to the supplementation concentrations that were studied that is 2 mmol/kg, 3 mmol/kg and 5 mmol/kg.

To validate that the slight compositional change did not impact the pace of vitamin C degradation, both matrices were carefully intermixed with ascorbic acid. The stability of vitamin C in the respective matrix was then examined under the study's reference condition. No difference was observed between the whole apple purée and its serum. A rate constant k_{app} of $30 \pm 2 \times 10^{-4}$ mmol kg^{-1} min^{-1} was determined for whole apple purée and of $31 \pm 2 \times 10^{-4}$ mmol kg^{-1} min^{-1} for the apple purée serum (in the following section, determination of rate constants is explained). The apple purée serum was retained for further studies as allowing more reliable homogenization and presenting only small differences in potentially impairing or protecting compositional properties.

4.2 Model discrimination and determination of rate constants

In literature, zero and first order models are often used to describe linear and exponential relationships of time dependent curves. With a view to gather quantitative data and classifying the significance of different factors, experimental data was fitted to the zero and first order model. Model discrimination was carried out at the study's reference conditions by comparison of Akaike information criteria (AIC). A degradation percentage of at least 70 %, as recommended by van Boekel (2009) for accurate model discrimination, was respected. Akaike information criteria were of -7.3 for the zero order model and 2.4 for the first-order model. This indicated better fit for the zero order model since the value is inferior to that of the first order model. The experimental data (points), the corresponding model (line) and the respective residuals for the two models are depicted in Figure 30.

The fitting of a zero order model looks visually acceptable although the fit at the beginning is not ideal but still admissible. Acceptable fit is corroborated by equally distributed residuals. In contrast, for the first order model, the fit at the end of the kinetics was not good and the residuals were not equally distributed.

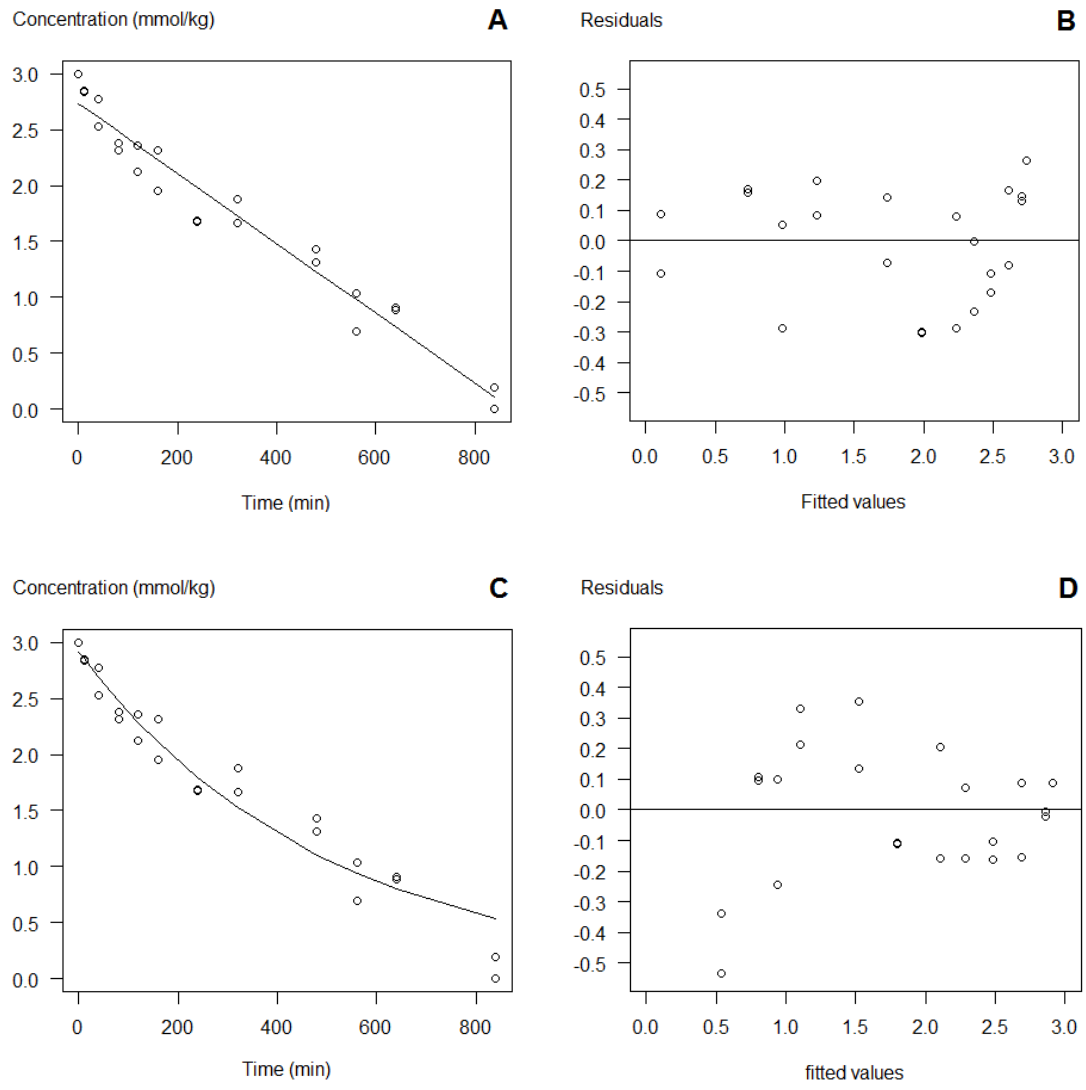


Figure 30: Model discrimination at the study's reference conditions

(apple purée serum, 3 mmol/kg vitamin C, 80 °C, 1.5 mL filling volume).

Model fit (line) to experimental data (points).

(A) Zero-order model

(C) First-order model

(B) Residuals of zero-order model

(D) Residuals of first-order model

4.3 pH impact

Firstly, pH dependence of vitamin C degradation was tested in citrate-phosphate buffer at pH 3.5, 5.5 and 7.5 (Figure 31 A, Table 9). Ascorbic acid was the most stable at pH 3.5 with a rate constant of $32 \pm 2\ 10^{-4}$ mmol kg^{-1} min^{-1} and the least stable at pH 5.5 with a rate constant of 70 ± 5 mmol$kg^{-1}min^{-1}$. Small difference between pH 3.5 and 7.5 was observed visually with slightly less stability at pH 7.5. There was however no difference when rate constants were compared ($32 \pm 2\ 10^{-4}$ mmol kg^{-1} min^{-1} vs. $35 \pm 3\ 10^{-4}$ mmol kg^{-1} min^{-1}). Wilson et al. (1995) observed no difference between rate constants in ethanoic buffer at pH 1.0 and 2.0 but when pH was changed to 3.9, 5.0 or 9.0. This can be supposed to be due to higher susceptibility of the monoionic form of ascorbic acid, dominant at pHs between its first pK_a (4.3) and its second pK_a (11.8). However, Rojas and Gerschenson (1997) observed that the stability of ascorbic acid depends on the used acid. They reported that ascorbic acid degrades faster in model solution acidified with phosphoric acid than with citric acid at 80°C. Different interaction between trace metals, ligands and oxygen might therefore have been responsible (Harel, 1994). The result of this study may thus have been a consequence of the citrate-phosphate buffer. The highest stability was observed at pH 3.5 which can be ascribed to dominance of the fully protonated form of ascorbic acid at a pH under its first pK_a. In addition, the proportion of citric and phosphate molecules changed with pH. The difference between pH 5.5 and 7.5 might thus be due to a change of ligands and their respective complex formation with trace metals and oxygen.

Table 9: Rate constants obtained by applying zero order model on vitamin C degradation kinetics under different extrinsic and intrinsic condition.

(kapp: apparent rate constant, conc.: concentration, temp.: temperature)

Medium	pH	k_{app} (10^{-4} mmol kg^{-1} min^{-1})	Conc. (mmol/kg)	Temp. (°C)	Filling volume (mL)
Phosphate-citrate buffer	3.5	32±2	3	80	1.5
Phosphate-citrate buffer	5.5	70±5	3	80	1.5
Phosphate-citrate buffer	7.5	35±3	3	80	1.5
Apple purée	3.5	30±2	3	80	1.5
Apple purée serum	3.5	31±2	3	80	1.5
Apple purée serum	7.5	29±4	3	80	1.5
Carrot purée	5.5	24±2	3	80	1.5
Apple purée serum	3.5	32±2	2	80	1.5
Apple purée serum	3.5	33±2	5	80	1.5
Apple purée serum	3.5	6±0	3	40	1.5
Apple purée serum	3.5	11±1	3	50	1.5
Apple purée serum	3.5	29±3	3	60	1.5
Apple purée serum	3.5	28±1	3	70	1.5
Apple purée serum	3.5	61±3	3	80	0.5
Apple purée	3.5	61±5	3	80	0.5
Apple purée serum	3.5	42±2	3	80	1.0

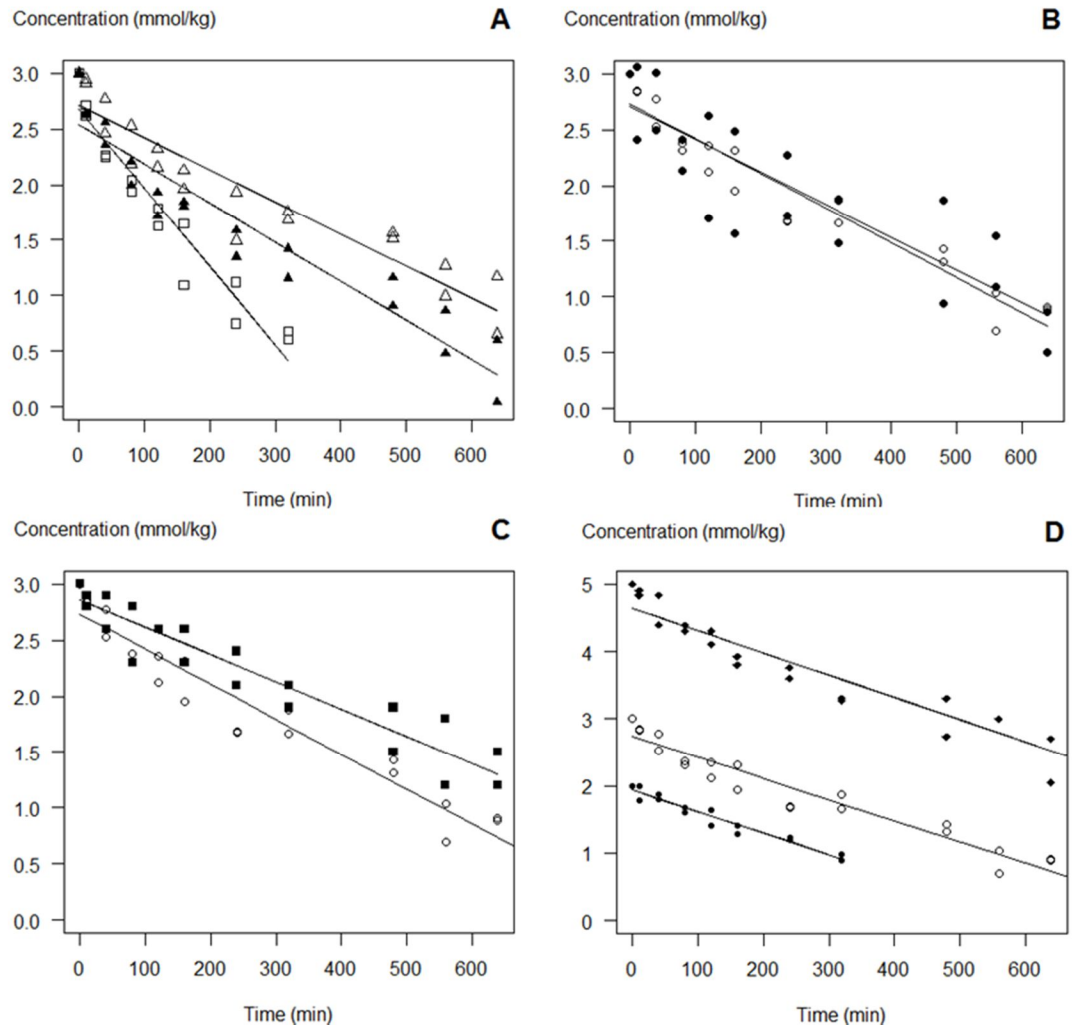


Figure 31: Effect of changing intrinsic factors on degradation of vitamin C.

A: Model solution: △ pH 3.5, □ pH 5.5 vs. ▲ pH 7.5;

B: Apple purée serum: ○ pH 3.5 vs. ● pH 7.5;

C: ○ apple purée serum vs. ■ carrot purée;

D: Different initial ascorbic acid concentration in apple purée serum: ● 2 mmol/kg, ○ 3 mmol/kg vs. ◆ 5 mmol/kg

To get a better idea of vitamin C stability as a function of pH in a real food matrix, the pH of the apple purée serum was increased by sodium hydroxide. Degradation curves and rate constants at natural pH of the apple purée serum and at a pH of 7.5 were subsequently compared (Figure 31 B, Table 9). Uncertainty of values was quite high at pH 7.5 with a rate constant of 29 ± 4 mmol kg^{-1} min^{-1} and on this basis, no difference was observed, neither visually nor by comparing rate constants.

In carrot purée, exhibiting a natural pH of 5.5 (Figure 31 C, Table 9), vitamin C was slightly more stable than in the apple purée serum which was observed visually as well as by comparing rate constants. The rate constant in carrot purée was 24 ± 2 mmol kg^{-1} min^{-1}. However, the origin for this difference is difficult to ascertain. Comparison with buffer solutions to elucidate the effect of pH is not reliable as the effect of pH, as shown above, depends enormously on the used buffer. Additionally, this origin might not exclusively arise from pH differences but also from the composition of the matrix. The stability is influenced by sugars, especially fructose, the prevalent sugar in many apples (Rojas & Gerschenson, 1997; Suni, Nyman, Eriksson, Bjork, & Bjorck, 2000). Polyphenols are reported to exhibit a protective effect at least at 37 °C compared to drinking water (Miller & RiceEvans, 1997). Antagonistic effects are probable, that is a protective and impairing effect of other naturally present components in food matrices. Observed degradation rates in apple purée serum and carrot purée were very close at 80°C in spite of the different compositions.

4.4 Concentration effect

Supplementation concentration depends on particular needs of consumer groups. Establishing the effect of concentration changes on degradation rates is therefore crucial. As expected from a zero order reaction, no difference was observed between the rate constants that were obtained by starting with different initial concentrations (Figure 31 D, Table 9). In contrast to this result, Oey et al. (2006) have reported on a concentration dependence of aerobic and anaerobic rate constants determined by applying a biphasic model which was based on a first order model and differentiated between aerobic and anaerobic degradation. Under their conditions, the degradation of vitamin C was fast at the beginning and then stagnated. Since they used glass vials with rubber septum oxygen availability in

the system may have been limited and explain the diverging results. A limited amount of oxygen and consequently a lower oxygen-to-ascorbic acid concentration-ratio when ascorbic acid concentration was high may explain the concentration dependence that they observed. A mathematical artifact may also have been involved, as they compared C/C_0, which mechanically increased the slopes for lower C_0. In addition vitamin C degraded linearly (concentration vs. time) in this study, which can be assumed to be due to constant oxygen contents in the system for transformation of ascorbic acid. Coherently, perforation of tubes caps did not lead to an enhancement of degradation. Wilson et al. (1995) determined rate constants by applying a first order model on graphs where the heat flow (transformed by the natural logarithm) was plotted against time. These rate constants were not affected by the concentration of ascorbic acid. As they worked at 25 °C dissolved oxygen availability is high and the chemical conversion might depend only on the activation energy.

4.5 Temperature dependence

Temperature dependence was studied between 40°C and 80°C (Figure 32 A). For reheating and warm-keeping of food especially the range 60-80 °C is important to ensure food safety that is to limit microbial growth. In this temperature range, rate constants did not differ (Table 9) in contrast a marked influence of temperature below 60 °C. Arrhenius equation was not employed as rate constants stayed constant between 60-80°C. Dhuique-Mayer et al. (2007) determined an activation energy of 36 kJ/mol in citrus juice in for the temperature range 50-100°C. Difference between the curves at 70 °C and 80 °C was however small and degradation was only followed at the very beginning with a limited number of points. In strawberries and raspberries, ascorbic acid degradation is only slightly temperature dependent in the range 80-90 °C (Verbeyst et al., 2013). The apparent stagnation of rate constants in the present study may hence indicate that energy supply was not the limiting factor but probably oxygen availability.

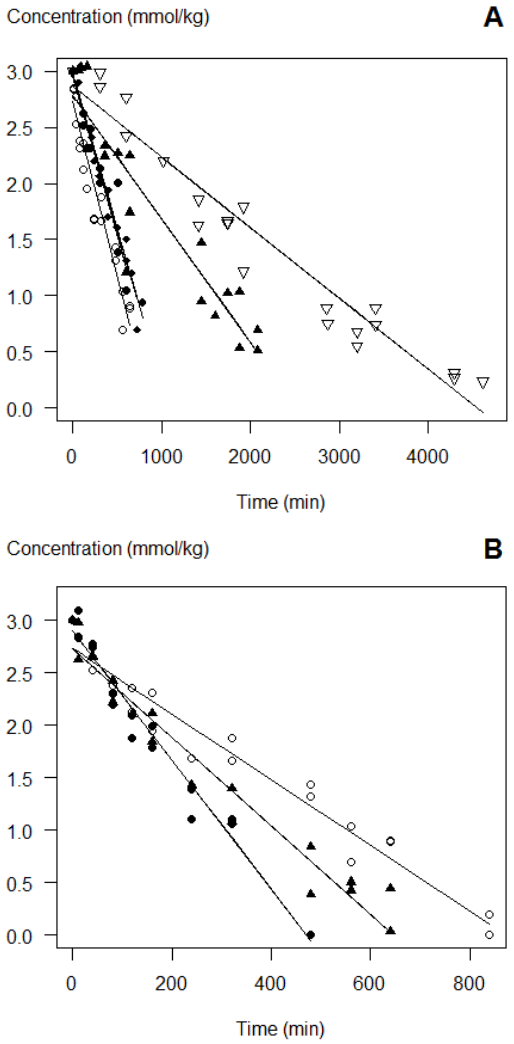


Figure 32: Effect of changing extrinsic factors on degradation of vitamin C.

A: Temperature dependence: ▽ 40°C , ▲ 50°C , ● 60°C , ◆ 70°C vs. ○ 80°C

B: Effect of the filling volume: ● 0.5 mL, ▲ 1.0 mL vs. ○ 1.5 mL

4.6 Impact of the surface-to-volume ratio

The influence of oxygen accessibility was studied by comparing three different filling volumes of experimental tubes and thus three different surface-to-volume ratios. Changing the surface-to-volume ratios had a major impact on vitamin C degradation rate in the temperature range 60-80°C (Figure 32 B, Table 9). The rate constant was approximately doubled by decreasing the filling volume of the tube from 1.5 to 0.5 mL. As whole apple purée contains more soluble fibers, potentially influencing

diffusion of ascorbic acid and oxygen in the medium and limiting convection, the filling volume was also changed for kinetics in whole apple purée (Table 9). Idem, vitamin C degraded faster when filling volume was smaller. Furthermore, no difference between the apple purée serum and the whole apple purée was observed for the smaller filling volume either. The causal origin of the impact of surface-to-volume ratio can arise only from different oxygen availabilities as other factors remained equal. Perforating tubes when filling volume was high did not increase the degradation rate in this study. Oxygen replenishment could thus be excluded. Ascorbic acid at 20°C degrades faster near the surface in agar gel, which was associated to higher amounts of dissolved oxygen in regions near the surface (Penicaud et al., 2011). Kinetics of vitamin C obtained by withdrawal of aliquots at the bottom and the surface of the same tube were not different. Longer diffusion distances to the surface when filling volume was high may be a possible explanation for the observed effect. Surface exposition appeared to be of crucial importance.

5. Conclusion

Comparison of vitamin C degradation in different conditions emphasized the crucial rule of extrinsic factors in a real food matrix. The surface-to-volume ratio was the factor that had the greatest impact if only the temperature range 60-80°C, which is usually sighted for warm holding of food, was considered. Adapted geometry of recipients might hence reduce losses during reheating. Temperature had only an impact on degradation in the range 40-60°C implying a mechanism change above 60°C. Oxygen availability in the medium decreases with temperature and oxidation reactions and might have been the limiting factor in apple purée serum between 60-80°C, as further supply of energy did not increase anymore the degradation pace. The common used Arrhenius equation to determine the activation energy (E_A) was consequently not applicable. Only a slight difference has been observed between apple purée and carrot purée as supplementation matrix and thus both matrices are likewise appropriate for supplementation. pH seems to play a minor role in real food matrices. Furthermore, degradation rate of ascorbic acid appeared independent of initial concentration making concentration adaptions easily predictable if other factors stay equal.

6. Acknowledgement

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Chapter II

This chapter is an accepted paper which has been submitted to the journal "LWT- Food Science and Technology". Authors were Anna-Lena Herbig, Jean-François Maingonnat and Catherine M. G. C. Renard. The article is entitled "Oxygen availability in model solutions and purées during heat treatment and the impact on vitamin C degradation".

1. Abstract

Oxygen availability in different media during heat treatment (8 h at 80°C) and the related vitamin C loss was assessed. Dissolved oxygen in water containing 3 mmol/kg of ascorbic acid decreased initially and seemed to be replaced by oxygen from the headspace in the course of time, as oxygen values increased again. In apple purée and carrot purée in contrast, oxygen was depleted within 60 minutes. Vitamin C in ultrapure water was stable even in the presence of oxygen. A trigger seemed to be crucial to launch vitamin C degradation. Fe^{3+} ions added to water, but also the media Mc Ilvaine citrate-phosphate buffer (pH 3.5) or apple purée, initiated degradation. Adding Fe^{3+} ions to apple purée did not accelerate vitamin C degradation but shifted the equilibrium between ascorbic acid and dehydroascorbic acid to the latter. Oxygen deprivation stabilized completely vitamin C, independently of the medium tested. A temperature decrease to 70°C or 60°C, in contrast, had no effect on the degradation extent of vitamin C in water containing 20 µmol/kg Fe^{3+} ions but led to complete stability in apple purée.

Keywords: Dissolved oxygen, anaerobic condition, ascorbic acid, dehydroascorbic acid, reheating

2. Introduction

Vitamin C is well known for exhibiting beneficial health properties (Block, 1991; Gale, Martyn, Winter, & Cooper, 1995; Zandi et al., 2004). It is however susceptible to deterioration. In raw products, enzymes as for example polyphenol oxidase accelerate this reaction. Polyphenol oxidase catalyzes the oxidation of polyphenols which leads to consumption of ascorbic acid since it acts as reducer of generated quinones (Aka et al., 2013). At elevated temperatures, where enzymes are

inactive, degradation still proceeds in significant amounts. The aerobic degradation pathway of ascorbic acid proceeds faster than the anaerobic one, which only occurs to significant amounts over 120°C (Dhuique-Mayer et al., 2007; Oey et al., 2006; Verbeyst et al., 2013). The availability of oxygen is thus crucial for the fate of vitamin C at an intermediate temperature range.

Oxygen is soluble in water up to 100°C (Penicaud et al., 2012). Its saturation is temperature and matrix dependent. An increase of temperature, salinity or °Brix leads to a decrease of dissolved oxygen. Verbeyst et al. (2013) supposed that dissolved oxygen in strawberry and raspberry pastes in the range 80-120°C was quickly consumed and led to the plateau they observed in terms of vitamin C degradation, even though heat treatment continued. Oxygen concentrations were however not measured simultaneously to degradation. In agar gel, a gradient of oxygen and vitamin C concentration occurs at 20°C in the course of time with higher degradation near the surface (Penicaud et al., 2011). The role of dissolved oxygen in citrus juice on vitamin C degradation at 90°C was studied by nitrogen substitution of initial dissolved oxygen (Dhuique-Mayer et al., 2007). However, as gases' solubility is decreased by heat, it is very likely that in this study, the liberation counted also for nitrogen and thus the oxygen-to-nitrogen-ratio can be assumed to have changed in the headspace too. Hence, a lower vitamin C degradation rate which was associated to lower dissolved oxygen contents, was presumably rather a combination of headspace and dissolved oxygen depletion.

Up to now it is not known how fast oxygen is consumed at elevated temperatures and if it is replaced quickly from the headspace. In addition, other oxidizable components are present in real food products contributing to oxygen consumption. Dissolved oxygen measurements at higher temperatures were difficult to achieve in the past. Measurements at precise locations became possible due to development of oxygen sensors (Liebsch, Klimant, Frank, Holst, & Wolfbeis, 2000) that can be installed on recipients' walls and are specially calibrated for elevated temperatures.

Besides oxygen, other food oxidants such as Fe^{3+} ions interfere in the degradation of ascorbic acid. Fe^{3+} ions accelerate the oxidation of ascorbic acid to dehydroascorbic acid and the hydrolysis of dehydroascorbic acid to 2,3-diketogulonic acid, by which the vitamin activity is lost (Serpen &

Gökmen, 2007). This seems to result from of a redox reaction with ascorbic acid being oxidized and Fe^{3+} ions being reduced to Fe^{2+} ions. Fe^{2+} ions, in turn, react in the Fenton reaction with hydrogen peroxide (Choe & Min, 2005) which is formed during the oxidation of ascorbic acid (Boatright, 2016). However, it is not known if the concomitant presence of oxygen is necessary for the Fe^{3+} ion-caused oxidation. Under anaerobic conditions and in the presence of Cu^{2+} ions, the stability of ascorbic acid can almost completely be preserved in contrast to aerobic conditions where ascorbic acid degrades rapidly (Boatright, 2016).

The aim of the present study was to reveal oxygen availabilities in different media during heat treatment at 80°C and to understand the influence of Fe^{3+} ions, ascorbic acid concentrations and temperature. Furthermore, the linked vitamin C degradation was investigated.

3. Material and Methods

3.1 Chemicals and Food Matrices

2,2′-Bipyridyl, ascorbic acid, trichloroacetic acid, DL-dithiothreitol, Na_2HPO_4, $NaH_2PO_4xH_20$, N-Ethylmaleimide and citric acid monohydrate were from Sigma-Aldrich (Deisenhofen, Germany). *Ortho*-phosphoric acid (85%), Iron (III) chloride hexahydrate from VWR (Leuven, Belgium) and ethanol from by Fisher Scientific (Fair Lwan, NJ, USA).

Apple purée (brand: POUCE) and carrot purée (brand: HIPP) without added vitamin C were purchased in a local supermarket in Avignon/France.

3.2 Supplementation and heat treatment

Ascorbic acid supplementation was carried out in corning tubes. After addition of ascorbic acid, samples were vortexed thoroughly, then transferred to a beaker and preheated to the respective temperature of interest while stirring. When temperature was achieved, the sample was immediately conveyed to a double walled system (Société Legallais, Montferrier-sur-Lez, France, Figure 33) which

was filled up to a height of 1 cm. The inner diameter of the vessel was 4.5 cm and the depth of the inner volume 2.7 cm. The double walled glass vessel was connected to a water bath (ED-19 Julabo, Seelbach, Germany) to maintain temperature of the medium. To avoid water evaporation, a plastic cap supported by silicon fat was put onto the top. As a consequence, the headspace became vapor saturated. Aliquots were withdrawn after the preheating step, corresponding to the initial value, and at the end of the heat treatment, and immediately deep-frozen (≤ -18°C).

When the effect of oxygen deprivation was evaluated, nitrogen was bubbled into a stirred and heated solution. Subsequently, the solution was transferred to a 12.5 cm glass tube (diameter: 1 cm) which was filled up to 4.5 cm under nitrogen flow. The tube was closed with a cap including a septum and allowing thus to maintain anaerobic conditions. The heat treatment was carried out by putting the glass tubes in a block heater (Stuart; Roissy Charles de Gaulle, France).

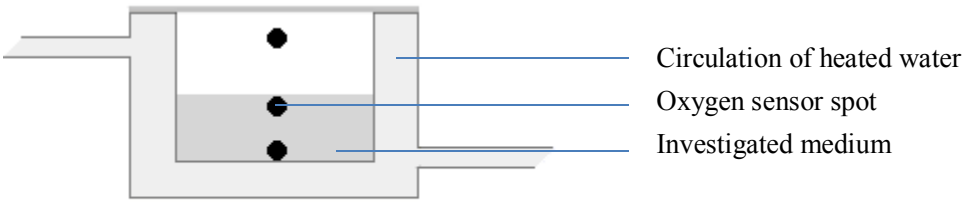


Figure 33: Double-walled vessel containing medium and three oxygen sensor spots.

3.3 Oxygen measurement

Planar oxygen sensors (Presens GmbH, Regensburg, Germany) with a diameter of 5 mm and calibrated up to a temperature of 80°C were stuck with silicon glue at the inner bottom, at a height of 1 cm and in the headspace region of the double walled glass vessel (Figure 33). Oxygen was measured from the outside of the vessel via a polymer optical fiber connected to a Fibox 4 Transmitter (Presens GmbH, Regensburg, Germany). Temperature in the vessel was measured by a sensor Pt100 (Presens GmbH, Regensburg, Germany) which was also linked to the Transmitter.

3.4 Vitamin C analysis

Vitamin C was quantified spectrophotometrically (Stevens et al. (2006). Approximately 500 mg aliquots were taken and absorption was measured at 525 nm on a spectrophotometer (Safas Xenius, Monaco).

4. Results

4.1 Treatment in water

Ultrapure water served as reference medium. After transferring the preheated solution to the double walled system, oxygen contents reached equilibrium in approximately 15 minutes (Figure 34 A), and then remained stable at 0.07 mmol/L in all three locations until the end of the measurement time. The oxygen content is in the range reported by Penicaud et al. (2012) for ultrapure water at 80°C. Oxygen equilibrium in the headspace and the medium was attained at the same pace.

In water containing 3 mmol/kg of ascorbic acid, oxygen contents dropped as in ultrapure water at the beginning of measurements (Figure 34 B). The decrease in the liquid was however higher than in the headspace, which can be ascribed to oxygen consumption by ascorbic acid. Oxygen was not entirely consumed even though ascorbic acid was in molar excess compared to oxygen (Figure 34 B). Since oxygen concentrations increased again, oxygen consumption and diffusion from the headspace into the medium seemed to compete and to lead to a dynamic equilibrium. In addition, as the oxygen consumption rate at the bottom and the surface of the medium was not significantly different, oxygen diffusion appeared to be a continuous and fast process at 80°C. The headspace oxygen level was however not reached again in the medium within the 8 h.

Furthermore, in spite of oxygen consumption and increase of dehydroascorbic acid, the overall vitamin C content did not decrease (Figure 34). Buettner (1988) observed also high stability of ascorbic acid when the medium was completely deprived of metals.

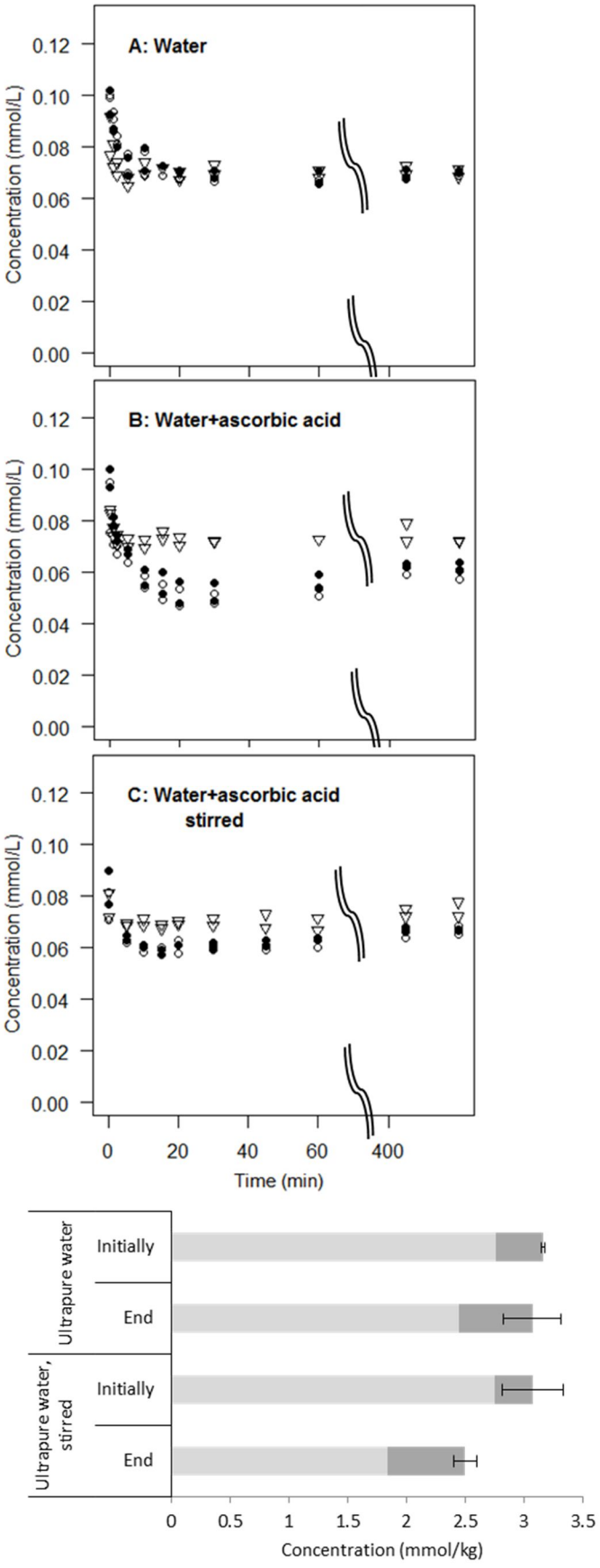


Figure 34: Oxygen time course at 80°C

in the ▽ headspace, and in the ○ surface and the ● bottom region of the medium.
A: Water
B: Water containing ascorbic acid (3 mmol/kg)
C: Stirred water containing ascorbic acid (3 mmol/kg)

And related vitamin C loss illustrated as bar plots. Light grey: ascorbic acid, dark grey: dehydroascorbic acid.

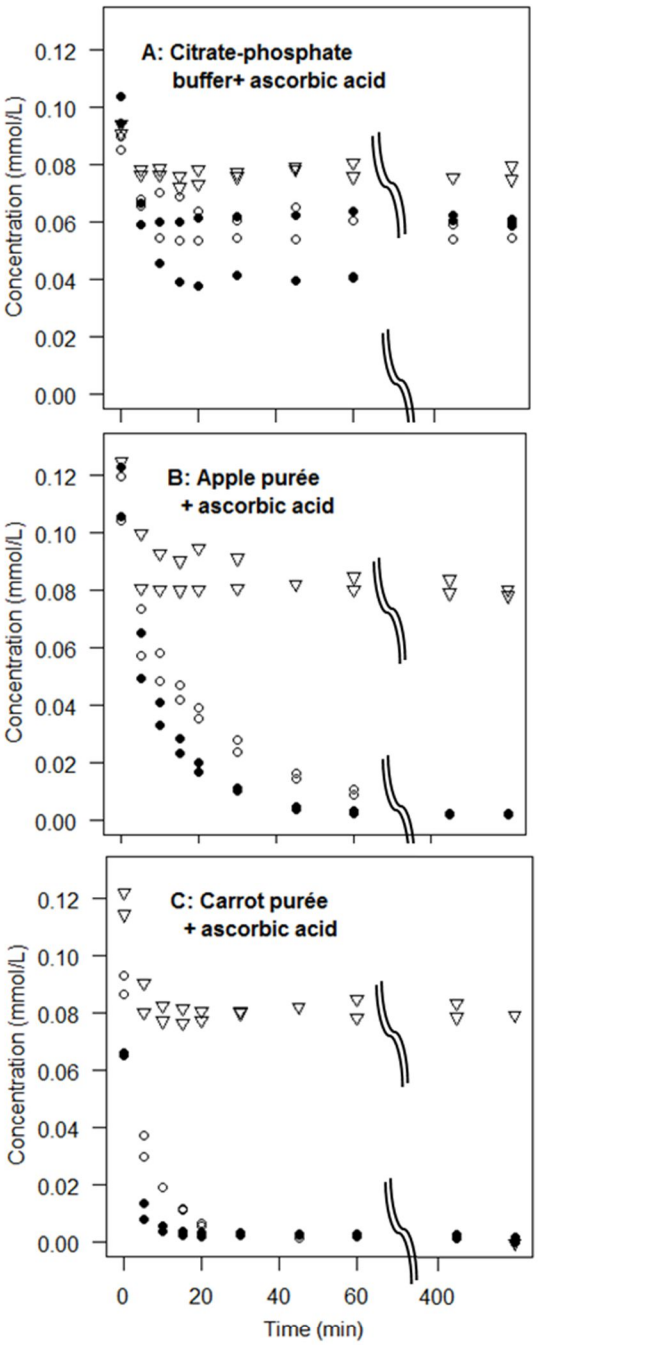
Subsequently, the experiment was repeated while stirring of the solution. Oxygen values decreased less at the beginning but still to a lower level than in the headspace (Figure 34 C). Oxygen was replaced more easily when the contact to headspace was increased. A vitamin C loss of 20 % was found.

4.2 Buffer and food matrices

Also in non-stirred Mc Ilvaine citrate-phosphate buffer (pH 3.5) that contained 3 mmol/kg of ascorbic acid, oxygen was more depleted in the medium compared to the headspace (Figure 35 A). A vitamin C loss of approximately 50 % was observed (Figure 35). The elevated variability of oxygen levels was also reflected by higher standard deviations of vitamin C contents. As reported by Buettner (1988), trace metals are detectable in many buffer solutions but not in double-distilled water, and might thus be responsible for vitamin C degradation in Mc Ilvaine citrate-phosphate buffer which was used in this study.

Oxygen decreased within 60 minutes down to anaerobic conditions in apple purée which was supplemented with 3 mmol/kg of ascorbic acid, and did not increase again until the end of experiment (Figure 35 B). Van Bree et al. (2012) reported that oxygen levels in the headspace decreased during vitamin C degradation under their conditions, which was however not the case here indicating that oxygen could enter from the outside. The oxygen decrease in the medium was slightly slower near the surface compared to the bottom but still proceeded within 60 minutes and down to anaerobic values. The complete elimination of oxygen may be caused by additional oxidation reactions of other components in the apple purée such as polyphenols.

Furthermore, even if oxygen was consumed entirely, 30 % of vitamin C was lost. This might be explained by oxidation due to a redox-reaction with polyphenols (Aka et al., 2013; Bradshaw et al., 2011). In this reaction, o*rtho*-quinones are reduced to the dihydroxyl corresponding molecule while ascorbic acid is oxidized to dehydroascorbic acid. This hypothesis was corroborated by the absence of a browning reaction at the very top surface when 3 mmol/kg of ascorbic acid was added (Supplementary data, Figure 48).



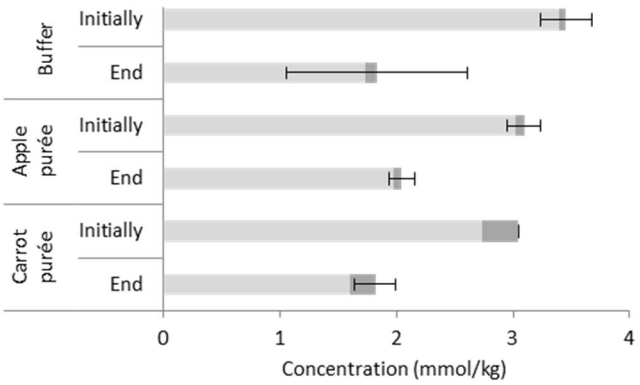


Figure 35: Oxygen time course at 80°C

in the ▽ headspace, and in the ○ surface and the ● bottom region of medium containing 3 mmol/kg ascorbic acid.
A: Citrate-phosphate buffer
B: Apple purée
C: Carrot purée

And linked vitamin C loss depicted as bar plots. Light grey: ascorbic acid, dark grey: dehydroascorbic acid.

In carrot purée, a decrease to anaerobic conditions, already within 20 minutes, was observed (Figure 35 C). As in apple purée, a slightly slower decrease was observed near the surface compared to the bottom. About 30 % of vitamin C was lost (Figure 35).

4.3 Impact of Fe^{3+} ions

Oxygen behavior was firstly studied in a blank consisting in water with 20 µmol/kg Fe^{3+} ions. As in ultrapure water, oxygen contents in all three oxygen sensor locations decreased in the first minutes down to one stable oxygen level which was of 0.08 mmol/L (Supplementary data, Figure 49).

Subsequently, the concentration effect of Fe^{3+} ions on oxygen availability and vitamin C stability was studied in water with 3 mmol/kg of ascorbic acid in the presence of three different Fe^{3+} concentrations (20, 100, 200 µmol/kg). Oxygen decreased the fastest when the Fe^{3+} ion concentration was of 100 µmol/kg and the slowest at a concentration of 200 µmol/kg (Figure 36).

The oxygen consumption time curve for water containing 20 µmol/kg Fe^{3+} ions was in between the two others. The difference of oxygen consumption rates was small but a similar trend was found for the corresponding vitamin C degradation levels. In the presence of a Fe^{3+} ion concentration of 20 and 200 µmol/kg, degradation levels were lower than in the presence of 100 µmol/kg Fe^{3+} (Figure 36). Higher oxygen concentrations and less degradation in the presence of 200 µmol/kg compared to 100 µmol/kg might be due to a changed equilibrium of Fenton reactions. On the one hand, Fe^{3+} ions accelerate ascorbic acid oxidation (Khan & Martell, 1967a), but on the other hand when superoxide anions encounter Fe^{3+} ions, they are inactivated by an electron transfer resulting in Fe^{2+} and molecular oxygen (Choe & Min, 2005).

Dehydroascorbic acid contents increased markedly compared to other media tested. This increase was already found at the beginning indicating fast reaction of Fe^{3+} ions (Figure 36). The accelerated oxidation to dehydroascorbic acid had however no influence on the overall vitamin C content (AA+DHA) at the beginning, since no significant degradation was found. The hydrolysis of dehydroascorbic acid proceeded to a significant extent, only in the course of time and might be dependent on the production of reactive oxygen species. Acceleration of the oxidation of ascorbic acid

to dehydroascorbic acid as well as of the hydrolysis of dehydroascorbic acid by Fe^{3+} ions was also ascertained by Serpen & Gökmen (2007).

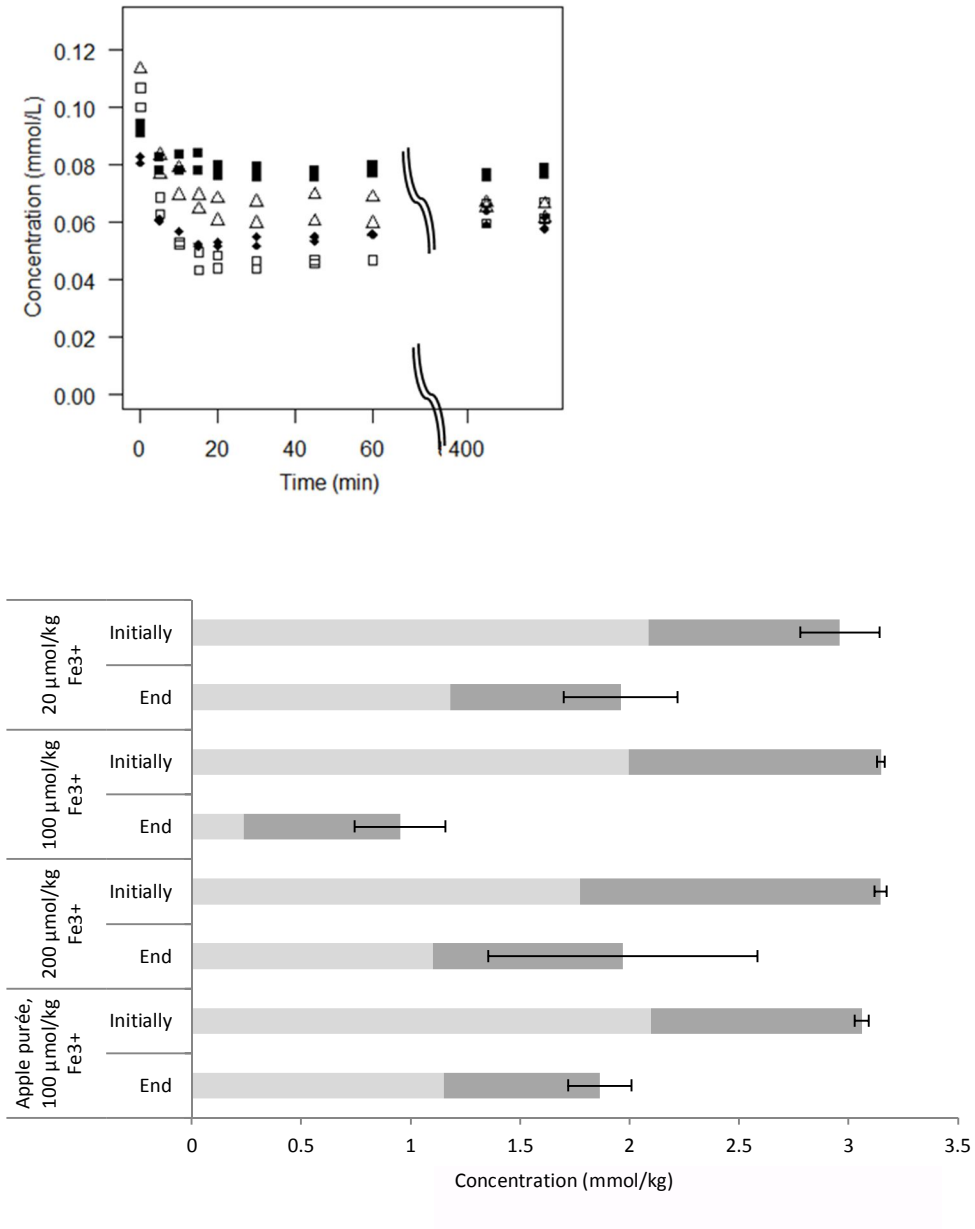


Figure 36: Oxygen time course at 80°C

in the ■ headspace region and in water containing ascorbic acid (3 mmol/kg)

and ● 20, □ 100 or △ 200 µmol/kg Fe^{3+} ions

Measured at the bottom respectively.

Related vitamin C loss illustrated as bar plots. Light grey: ascorbic acid, dark grey: dehydroascorbic acid.

With a view to understand the role of Fe^{3+} ions in a real food matrix, 100 µmol/kg of Fe^{3+} ions were added to apple purée. As in apple purée without added Fe^{3+} ions, oxygen was depleted within 60 minutes (Supplementary data, Figure 50). The equilibrium between ascorbic acid and dehydroascorbic acid was, like in water containing Fe^{3+} ions, already initially shifted to dehydroascorbic acid. At the end, the vitamin C loss in apple purée with Fe^{3+} ions was not elevated compared to apple purée without additional Fe^{3+} ions (Figure 36). As oxygen was depleted in apple purée, an oxygen lack might have caused this non-effect of Fe^{3+} ions.

4.4 Impact of the concentration of ascorbic acid

The impact of the concentration of ascorbic acid was studied in water with 20 µmol/kg Fe^{3+} ions in the presence of three different ascorbic acid concentrations (3, 6 and 12 mmol/kg). The depletion of oxygen did not increase with the concentration of ascorbic acid (Supplementary data, Figure 51) and relative vitamin C degradation that is, the degradation expressed as percentage of the initial value, did not differ within the uncertainty of values (Figure 37). This is in accordance to the result of Oey et al. (2006) who report that concentration of ascorbic acid does not influence the degradation pace of the aerobic pathway.

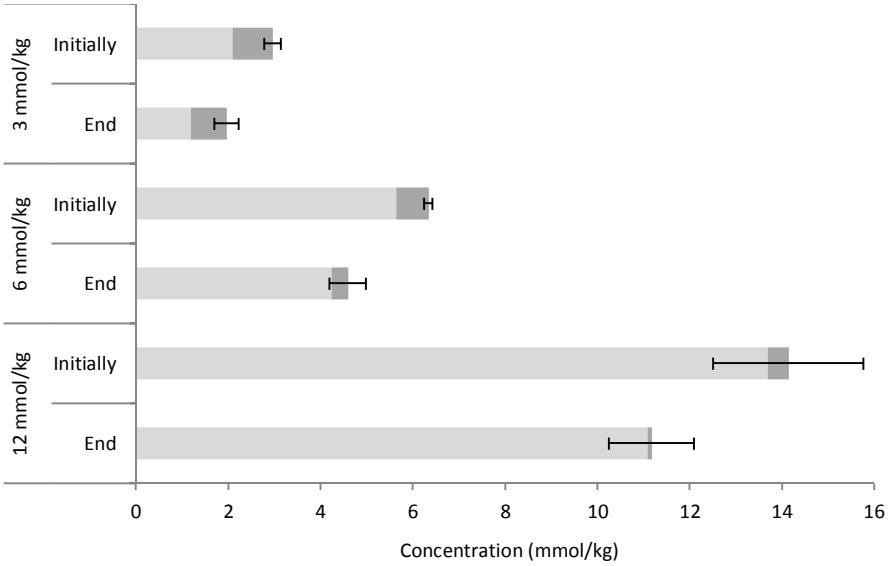


Figure 37: Effect of the initial concentration of ascorbic acid on its degradation extent at 80°C.

(light grey: ascorbic acid, dark grey: dehydroascorbic acid)

4.5 Anaerobic vs. aerobic degradation

To study the effect of oxygen deprivation, the experimental set-up was changed to glass tubes which permitted to maintain an air-tight system. The experiment was first conducted under aerobic conditions, that is without oxygen substitution, in water containing 20 µmol/kg of Fe^{3+} and 3 mmol/kg of ascorbic acid. Under this condition, vitamin C degraded significantly after 6 h at 80°C (Figure 38). With a view to verify if Fe^{3+} ions interact with ascorbic acid also in the absence of oxygen, oxygen was substituted by nitrogen. No degradation occurred (Figure 38). Fe^{3+} ions did not lead directly to degradation of ascorbic acid. Neither Fe^{3+} ions nor oxygen alone (complete stability of ascorbic acid was observed in ultrapure water, Figure 34) were able to trigger ascorbic acid degradation. Their combination appeared to be crucial. A marked protective effect, when oxygen is replaced by argon in a solution containing Cu^{2+} ions, was also observed by Boatright (2016).

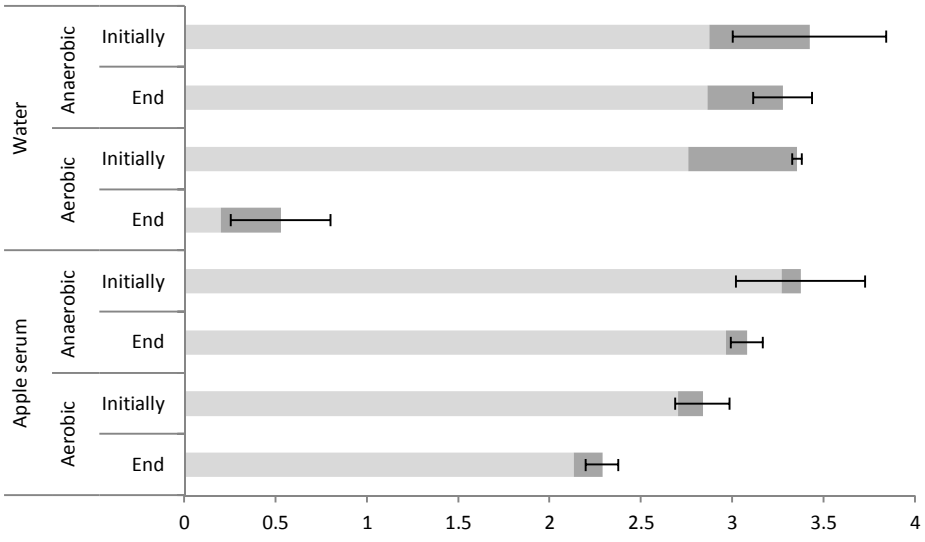


Figure 38: Effect of oxygen deprivation on the degradation extent of vitamin C at 80°C in water containing 20 µmol/kg Fe^{3+} ions and in apple serum.

Subsequently, the effect of oxygen deprivation was assessed in the supernatant of centrifuged apple purée. This medium was chosen as oxygen is difficult to eliminate from viscous apple purée and vitamin C degradation proceeds at the same pace in these two media (Chapter I). Like in water with 20 µmol/kg Fe^{3+} ions, vitamin C degraded under aerobic but not under anaerobic conditions. Oxygen seemed, also in apple purée, to be fundamental to initiate vitamin C degradation.

4.6 Influence of temperature

The impact of decreasing temperature on oxygen values and related vitamin C degradation was studied in water containing 20 µmol/kg Fe^{3+} ions and in apple purée, both supplemented with 3 mmol/kg ascorbic acid. Decreasing temperature to 70°C or 60°C led, as expected (Penicaud et al., 2012), in the headspaces to increased oxygen contents compared to 80°C.

In water with 20 µmol/kg Fe^{3+} ions, similar oxygen behavior at 70°C and 60°C as at 80°C was observed. Oxygen levels first dropped and then increased again without reaching the level of the headspace (Supplementary data, Figure 52). Oxygen contents at the bottom and the surface were not significantly different. Oxygen stayed abundant over the whole period. Furthermore, vitamin C was degraded to the same extent at 70°C and 60°C compared to 80°C with a loss of 30 %.

As at 80°C, oxygen in apple purée was depleted more than in water at 70°C and 60°C (Figure 39). The oxygen decrease proceeded however slower at 70°C and 60°C compared to 80°C in apple purée. The equilibrium was reached within 120 minutes at 70°C and within 180 minutes at 60°C. A difference between oxygen contents at the bottom and near the surface was observed not only for the time interval in which oxygen decreased continuously, but also after having reached the plateau. At the bottom, anaerobic conditions were found, which was not the case for at the surface. This might indicate faster oxygen renewal from the headspace in the surface region relative to oxygen consumption. Consistently, Penicaud et al. (2011) observed an oxygen content gradient at 20°C in agar gel containing ascorbic acid. Even though oxygen was consumed, which might be due to oxygen consumption of polyphenols, the vitamin C loss was not significant; neither at 70°C nor at 60°C. Vitamin C degraded in apple purée only at 80°C.

Since a temperature decrease led only in apple purée and not in water containing Fe^{3+} ions to higher stability of vitamin C, it appeared that the heat-sensitivity was medium dependent.

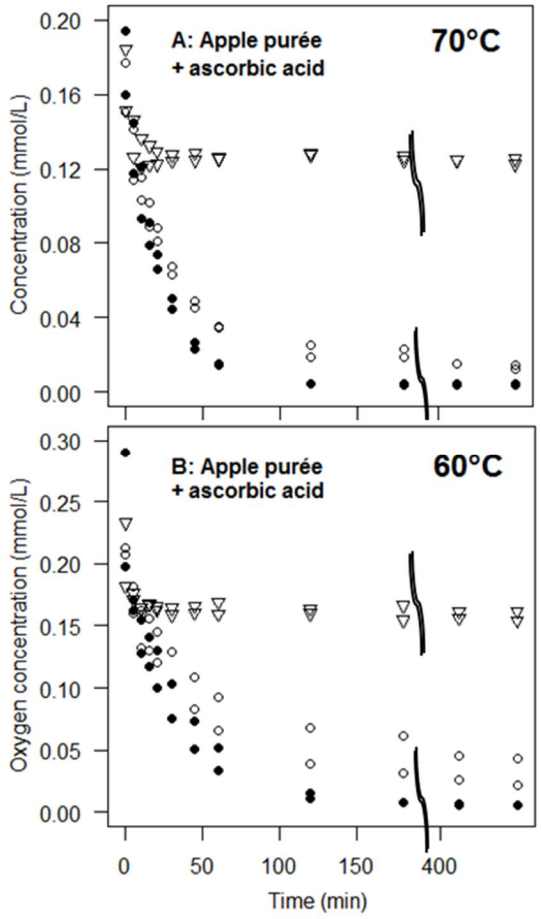


Figure 39: Temperature effect on oxygen contents in apple purée containing 3 mmol/kg of ascorbic acid.

Measured in the ▽ headspace, and in the ○ surface and ● the bottom region of the medium.

A: 70°C

B: 60°C

4.7 Convection and diffusion in the double walled system

To get an idea of mixing mechanisms that occur in the double envelope system and might contribute to homogenization of oxygen or vitamin C concentration, a spatula tip of methylene blue was put on the top of water and apple purée at 80°C respectively. Convection occurred in water which was indicated by inhomogeneous distribution of the colorant over time (Supplementary, Figure 53). However, spreading proceeded rapidly and thus a uniform coloration was obtained within 30 s. In the apple purée, in contrast, movement occurred only via diffusion (Supplementary, Figure 54). No flow marks were seen. The colorant disseminated slowly in circles around the point where it was set. Since

convection was absent in viscous apple and carrot purée, limited oxygen replacement might occur in such media.

5. Conclusion

Vitamin C kinetics in buffer solution should be carefully interpreted, especially if the results are related to real food products. Availability of oxygen and oxidation catalysts are crucial for ascorbic acid degradation and are not the same in water, buffer solution or food products. Vitamin C was stable for 8 h at 80°C in ultrapure water even though oxygen was present in excess. In citrate-phosphate buffer (pH 3.5), degradation occurred, which might be linked to trace metal ions. In apple and carrot purée, in turn, oxygen was consumed entirely within 60 minutes. Oxygen was not replaced again, not even near the surface. As vitamin C degradation was strongly linked to the availability of oxygen, the degradation mechanism in buffer and food products can be supposed to differ and explain why the heat sensitivity of vitamin C depended on the medium. Vitamin C was preserved completely by a temperature decrease to 60°C and 70°C in apple purée but not in water containing 20 µmol/kg Fe^{3+} ions. Fe^{3+} ions added to apple purée shifted the initial equilibrium of ascorbic acid to dehydroascorbic acid but did not, like in water, accelerate the total vitamin C degradation what might be linked to the limited oxygen availability in apple purée. For prediction of vitamin C losses, working with real food product appears to be indispensable.

6. Acknowledgement

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Chapter III

This chapter is planned to be published. Authors were Anna-Lena Herbig, Nicolas Delchier, Lisa Striegel, Michael Rychlik and Catherine M. G. C. Renard. It is entitled "Stability of 5-methyltetrahydrofolate supplemented in apple purée and carrot purée".

1. Abstract

5-Methyltetrahydrofolate, the naturally abundant folate vitamer, has been proposed as alternative to folic acid for supplementation. However, its stability is described to be less than that of folic acid, which was confirmed in this study. In formic acid buffer (pH 3.5), folic acid was preserved entirely after heating the solution for 3 h at 80°C. In contrast, 5-methyltetrahydrofolate was completely degraded in less than 15 minutes. By adding 2840 µmol/kg (or 50 mg/100 g) of ascorbic acid, stability of 5-methyltetrahydrofolate was attained for 3 h at 80°C. As in buffer, 5-Methyltetrahydrofolate added to apple purée degraded rapidly without addition of ascorbic acid. The protection duration of ascorbic acid depended on the added concentration. Degradation started after approximately 60 minutes when 570 µmol/kg (or 10 mg/100 g) of ascorbic acid was added, after 120 minutes with 1420 µmol/kg (or 25 mg/100 g) and was completely prevented by adding 2840 µmol/kg. When carrot purée was used as supplementation matrix, the stability of 5-methyltetrahydrofolate did not change when the same amount of ascorbic acid was added as had been used for apple purée. A temperature decrease to 70°C or 60°C, respectively, did not increase the stability of 5-methyltetrahydrofolate.

Keywords: folate, fortification, food matrix, degradation, impacts

2. Introduction

Folic acid supplementation is linked to a reduced risk of neural tube defects of newborns and became thus mandatory in the United States and other countries for cereal products (Eichholzer, Tonz, & Zimmermann, 2006; Grosse & Collins, 2007). In addition, insufficient intake of folates is associated with a higher colorectal cancer risk (Kim, 2003). Folic acid, a synthetic vitamer, is predominantly used for supplementation and is transformed in the body to naturally occurring forms. However, when

ingested in high doses, it also circulates in the unmetabolized form (Smith, Kim, & Refsum, 2008). Furthermore, folic acid supplementation might mask vitamin B_{12} deficiency and thus 5-methyltetrahydrofolate, being the naturally abundant form in the body, has been proposed as alternative for fortifications (Pietrzik, Bailey, & Shane, 2010a; Scaglione & Panzavolta, 2014).

Concerning its chemical structure, 5-methyltetrahydrofolate differs from folic acid by being reduced in position N(5), C(6) and C(7), N(8); and the hydrogen in position N(10) is substituted by a methyl group (Delchier et al., 2016; Scott, Rebeille, & Fletcher, 2000). This structural difference has an enormous effect on stability. 5-Methyltetrahydrofolate is highly susceptible to oxygen compared to folic acid. In absence of oxygen, 5-methyltetrahydrofolate is completely stable (Delchier, Ringling, Cuvelier, et al., 2014). The stability of 5-methyltetrahydrofolate under aerobic condition decreases with rising temperature (Indrawati et al., 2004; Nguyen et al., 2003; Oey et al., 2006) and depends on pH. It is more stable in neutral compared to acid medium (Indrawati et al., 2004; Liu et al., 2012). At equal pH, stability depends additionally on the buffer solution used (Indrawati et al., 2004; Paine-Wilson & Chen, 1979).

5-Methyltetrahydrofolate degradation is accelerated by fructose but not by glucose (Verlinde et al., 2010). The impact is independent of the sugar concentration in the range 1.6 mmol/L-1.5 mol/L and is linked to a new product that is formed by glycation of the exocyclic amino group of 5-methyltetrahydrofolate (Figure 40). This product is formed along with the three degradation products that are also generated in the absence of fructose (Figure 40). The acceleration of degradation by fructose is counteracted by ascorbic acid probably by reduction of the first oxidation product, 5-methyl-7,8-dihydrofolate.

The degradation of 5-methyltetrahydrofolate decreases proportionally to the ascorbic acid concentration (Liu et al., 2012; Oey et al., 2006). Oey et al. (2006) related the concentration of ascorbic acid necessary to protect 5-methyltetrahydrofolate to the initial oxygen concentration. The concentration of ascorbic acid that led to complete protection between 50°C and 170 °C for 15 minutes was claimed to be twice as high as the molar initial oxygen concentration. However, the relationship

can supposed to be valid only in the analyzed time range since ascorbic acid is also susceptible to degradation and thus the protection effect will be lost once that ascorbic acid is degraded.

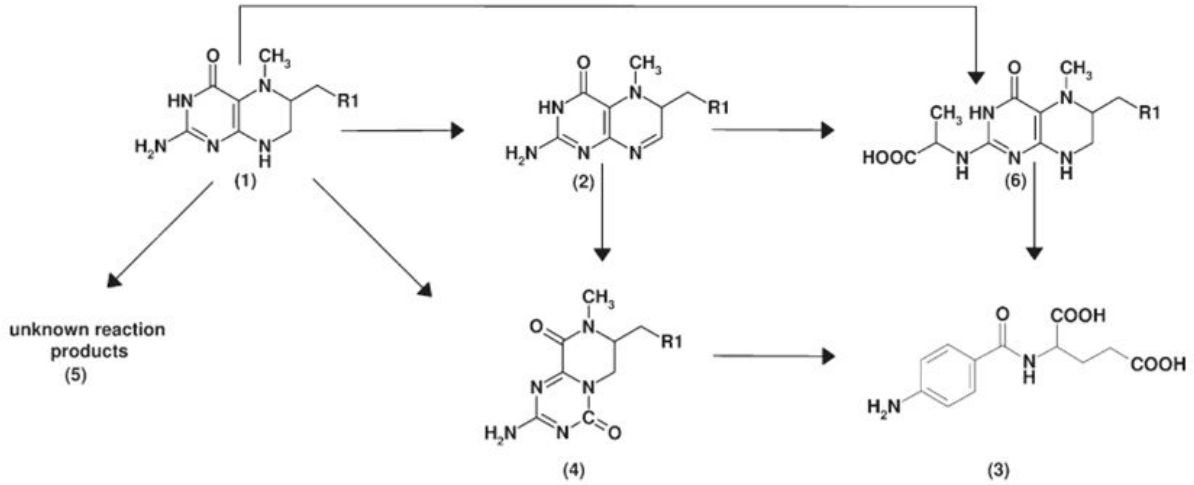

Figure 40: Degradation mechanism of 5-methyltetrahydrofolate in the presence sugars.

(1: 5-methyl-5,6,7,8-tetrahydrofolate, 2: 5-methyl-7,8-dihydrofolate, 3: p-aminobenzoyl glutamic acid, 4: 2-amino-8-methyl-4,9-dioxo-7-methyl-p-aminobenzoylglutamate-6,7,8,9-tetrahydro-4H-pyrazino(1,2-a)-s-triazine, 5 : unknown reaction products, 6 : N(2α)-[1-(carboxyethyl)]-5-methyl-5,6,7,8-tetrahydrofolic acid.)

Postulated and taken from Verlinde et al. (2010), adapted version.

Additionally, dissolved oxygen in real food products may be consumed also by other oxidizable compounds, which may change the relationship. For example, 50 mg/100 g of ascorbic acid increases the stability of 5-methyltetrahydrofolate in carrot juice but not in asparagus when heat treated for 40 minutes at 120 °C (Indrawati et al., 2004). The stability difference of 5-methyltetrahydrofolate in skim milk and soy milk has been linked to the different antioxidant capacities of the two food systems (Liu et al., 2012).

For the present study, apple purée and carrot purée were taken as supplementation matrices as they are usually less heat treated than cereal products and are well appreciated all over Europe. The objective was to find suitable conditions in the temperature range 60-80°C to impart high stability of 5-methyltetrahydrofolate in apple purée and carrot purée.

3. Material and Methods

3.1 Chemicals

2-(N-morpholino)-ethanesulfonic acid (MES) hydrate, formic acid, 2,2′-Bipyridyl, ascorbic acid, trichloroacetic acid, DL-dithiothreitol, Na_2HPO_4, $NaH_2PO_4xH_20$, N-Ethylmaleimide and citric acid monohydrate were purchased from Sigma Aldrich (Steinheim, Germany) and acetonitrile, methanol, ortho-phosphoric acid (85%), iron(III)chloride hexahydrate and ascorbic acid form VWR (Darmstadt, Germany). 2,3-Dihydroxybutan-1,4-dithiol was provided by Applichem GmbH (Darmstadt, Germany) and [$^{13}C_5$]-5-CH_3-H_4folate, [$^{13}C_5$]-folic acid, disodium hydrogen phosphate, potassium dihydrogen phosphate, sodium acetate trihydrate and acetic acid by Merck (Darmstadt, Germany). Sodium hydroxide and sodium chloride were obtained from J.T. Baker (Deventer, the Netherlands) and ethanol from Fisher Scientific (Fair Lawn, NJ, USA). 5-CH_3-H_4folate was purchased from Schircks (Jona, Switzerland) and folic acid from Sigma Aldrich (Steinheim, Germany).

3.2 Supplementation and thermal treatment

Formic acid buffer (0.1 %, v/v) was adjusted with sodium hydroxide to pH 3.5. Apple purée (brand: ALNATURA) and carrot purée (brand: HIPP) without additives were purchased at a supermarket in Freising/Germany. 5-Methyltetrahydrofolate and folic acid were dissolved in phosphate buffer (pH 7.0) and diluted with distilled water to a volume of 100 mL. Subsequently, 10 mL of this solution were added to 40 g of apple purée or carrot purée. Amber glass vials (4 mL) were filled with 2 mL of buffer solution or to the corresponding height in case of apple and carrot purée, and closed with screw caps. Thermal treatments were carried out in a silicon oil bath. After withdrawal, tubes were immediately deep-frozen (≤ -18°C). Before analysis, glass vials were put in a water bath for rapid defrosting. The first point of every time curve was omitted from heat treatment.

3.3 Folate analysis

The method of Ringling and Rychlik (2013) was employed with the modification that only the vitamer 5-methyltetrahydrofolate was used and ^{13}C- instead of ^{2}H-labeled standards were taken for quantification. Instrumental conditions were identical. Values were first calculated as calcium salt. The percental aberration of a fixed standard concentration of 200 µg/100 g was then calculated and was in average 7 ± 7 %. Each point of a time curve was subsequently corrected by the percental aberration of the first value to the standard concentration, to avoid parallelism of kinetics. The values were then transferred to their actual 5-methyltetrahydrofolate content by taking into consideration the calcium proportion, and converted to their molar concentration by respecting a molar mass of 459 g/mol.

3.4 Ascorbic acid analysis

Ascorbic acid quantification was carried out by the method of Stevens et al. (2006). Modifications were the following: approximately 500 mg of the food sample was taken for extraction. Absorption was measured at 525 nm on a spectrophotometer (Safas Xenius, Monaco).

4. Results

4.1 Natural folate concentration, extraction yield and test of homogeneity

Apples and carrots are food matrices that contain inherently low amounts of folates. Raw apples do not contain any measurable folates amounts and raw carrots only 19 µg/100 g folates (USDA, 21.7.2016). The amount of 5-methyltetrahydrofolate monoglutamate in the purchased apple purée was under the limit of quantification of the analytical method, which is of 0.22 µg/100 g (Ringling & Rychlik, 2013). The concentration of 5-methyltetrahydrofolate in carrot purée was 2.5 ± 0.1 µg/100 g. Natural amounts of 5-methyltetrahydrofolate in both matrices were thus negligible compared to the aimed supplementation level of 185 µg/100 g corresponding to 4.0 µmol/kg. Folic acid is a synthetic, not naturally occurring vitamer, and is thus not present in apple and carrots.

In order to verify homogeneity of supplementation preparations, apple purée was supplemented with folic acid. Recovery rates accounted to 107 ± 5 % and 109 ± 1 %, which were determined by analyzing four aliquots of two independently prepared supplementation mixtures. Extraction was thus quantitative and homogeneity was acceptable indicated by low standard deviations.

4.2 Stability in buffer solution and apple purée

Stability of folic acid and 5-methyltetrahydrofolate was first evaluated in formic acid buffer (0.1%, v/v, pH 3.5) heated at 80°C. The daily folate amount recommended by the European Food Safety Authority (EFSA) depends on the origin of folates since natural folates are less bioavailable than folic acid (EFSA, 2014). A dietary folate equivalent (DFE) is usually applied to convert folic acid amounts to the concentration that would be ingested if natural folates were absorbed. 1 µg DFE is defined as 1 µg food folate which in turn corresponds to 0.6 µg folic acid fortified to food. This conversion factor was considered when preparations were carried out and results in differing initial values depending on the used vitamer (Figure 41). Coherently to literature results (Delchier et al., 2016; Obroin et al., 1975; Paine-Wilson & Chen, 1979), folic acid was more stable than 5-methyltetrahydrofolate (Figure 41 A). Folic acid dissolved in formic acid buffer was completely stable at 80°C during 180 minutes, 5-methyltetrahydrofolate in turn degraded entirely within 15 minutes. The stability of folates depends on ions (Indrawati et al., 2004; Paine-Wilson & Chen, 1979) and hence direct comparison of degradation rates with literature results is not possible as other buffer solutions were used. However, degradation of 5-methyltetrahydrofolate occurred in the same time range as previously reported being a complete loss within some minutes (Obroin et al., 1975; Paine-Wilson & Chen, 1979).

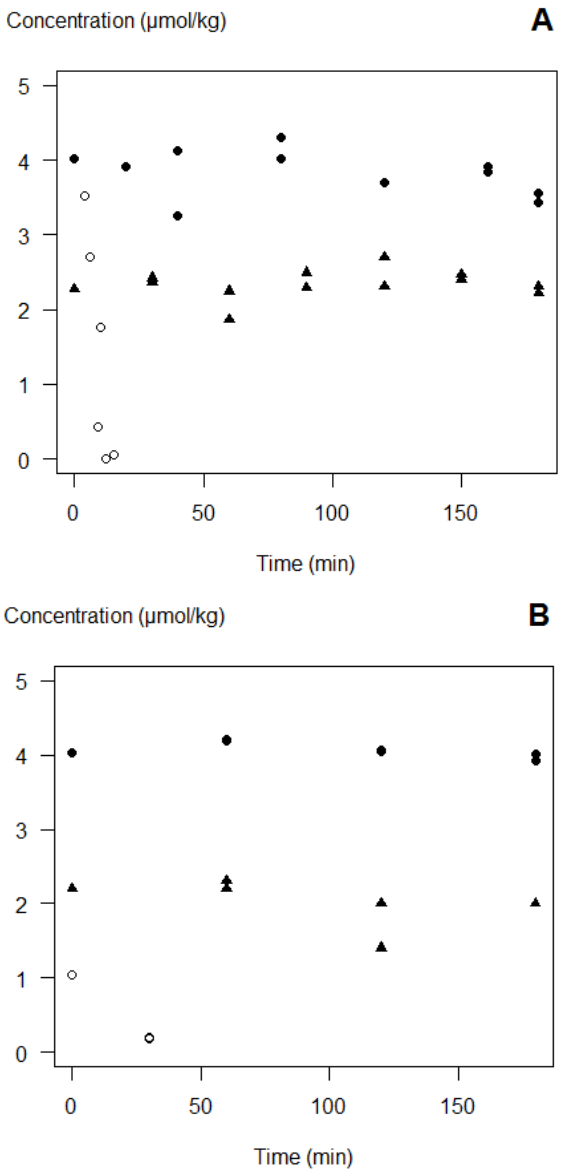

Figure 41: Degradation kinetics at 80°C in formic acid buffer and apple purée.

▲ Folic acid vs. ○ 5-CH_3-H_4folate and ● 5-CH_3-H_4folate in the presence of ascorbic acid (initial conc.: 2840 µmol/kg).

A: Formic acid buffer (0.1 %)

B: Apple purée

According to literature, entire stability of 5-methyltetrahydrofolate for 15 minutes up to 170°C can be obtained by adding ascorbic acid (Oey et al., 2006). In the present study, addition of 2840 µmol/kg of ascorbic acid induced complete stability of 5-methyltetrahydrofolate in formic acid buffer during three hours at 80°C (Figure 41 A). Apple purée contains fructose, which is known to accelerate degradation of folates (Verlinde et al., 2010), but also polyphenols that may potentially exhibit a protective effect as a result of their anti-oxidative properties. Therefore, the stability of folic acid and 5-methyltetrahydrofolate was also assessed in apple purée (Figure 41 B). As in formic acid buffer, folic acid was completely stable over the investigated time range and 5-methyltetrahydrfolate degraded rapidly (Figure 41 B). This is in accordance with the results of Mnkeni and Beveridge (1983) who ascertained low stability of 5-methyltetrahydrofolate in apple juice. When 2840 µmol/kg of ascorbic acid were added, complete stability of 5-methyltetrahydrofolate was also attained in apple purée. Natural present polyphenols in apple purée did not lead to the same stability as the addition of 2840 µmol/kg of ascorbic acid. Ascorbic acid protects 5-methyltetrahydrofolate completely in apple purée even in the presence of a high amount of fructose. This is coherent with the result of Verlinde et al. (2010) who studied the stability of 5-methyltetrahydrofolate (0.04 mmol/L) in the presence of fructose (0.04 mmol/L) and ascorbic acid (1.13 mmol/L) in a model solution which was heated for 45 minutes at 100°C.

5-Methyltetrahydrofolate is thus, in the presence of 2840 µmol/kg of ascorbic acid, equally suitable for supplementation as folic acid. Both vitamers withstood long periods of heating that can be encountered when food is reheated and kept warm for several hours. Up to now, flour products have exclusively been chosen for mandatory folic acid supplementation in the United States. However, folic acid, a fairly stable folate vitamer, is lost during the bread baking process (Gujska & Majewska, 2005). Neither folic acid nor 5-methyltetrahydrofolate in the presence of 2840 µmol/kg of ascorbic acid, were lost in apple purée under the reported conditions. Thus, with regards to stability, apple purée exhibits an advantageous supplementation matrix compared to flour that is baked to bread.

4.3 Concentration effect of ascorbic acid tested in apple and carrot purée

The amount of ascorbic acid (2840 µmol/kg) which was used for the assessment above, was added in excess compared to the content of 5-methyltetrahydrofolate (4.0 µmol/kg). It was also much higher compared to the concentration of dissolved oxygen which was not measured but is of 258 µmol/kg in water at 25°C and is probably even less under the studied conditions given the matrix and the temperature used (Penicaud et al., 2012). In the following, lower amounts of ascorbic acid were added to apple purée to verify if stability was still maintained. However, addition of neither 570 µmol/kg nor 1420 µmol/kg led to absolute stability of 5-methyltetrahydrofolate within the investigated time range as it had been observed for a concentration of 2840 µmol/kg (Figure 42 A). When adding 570 µmol/kg of ascorbic acid, degradation started within 60 minutes and amounted to 30 ± 1 % after 180 minutes and in the case of 1420 µmol/kg initiated within 120 minutes and reached 17 ± 3 % after 180 minutes. However, when degradation started, the depletion pace at both ascorbic acid concentrations was still slower than when no vitamin C was added. To examine if the loss of complete preservation of 5-methyltetrahydrofolate was associated with a depletion of ascorbic acid, the concentration of the latter was followed over the investigated time period (Figure 42 B). However, quite high amounts of ascorbic acid were still present when degradation of 5-methyltetrahydrofolate started.

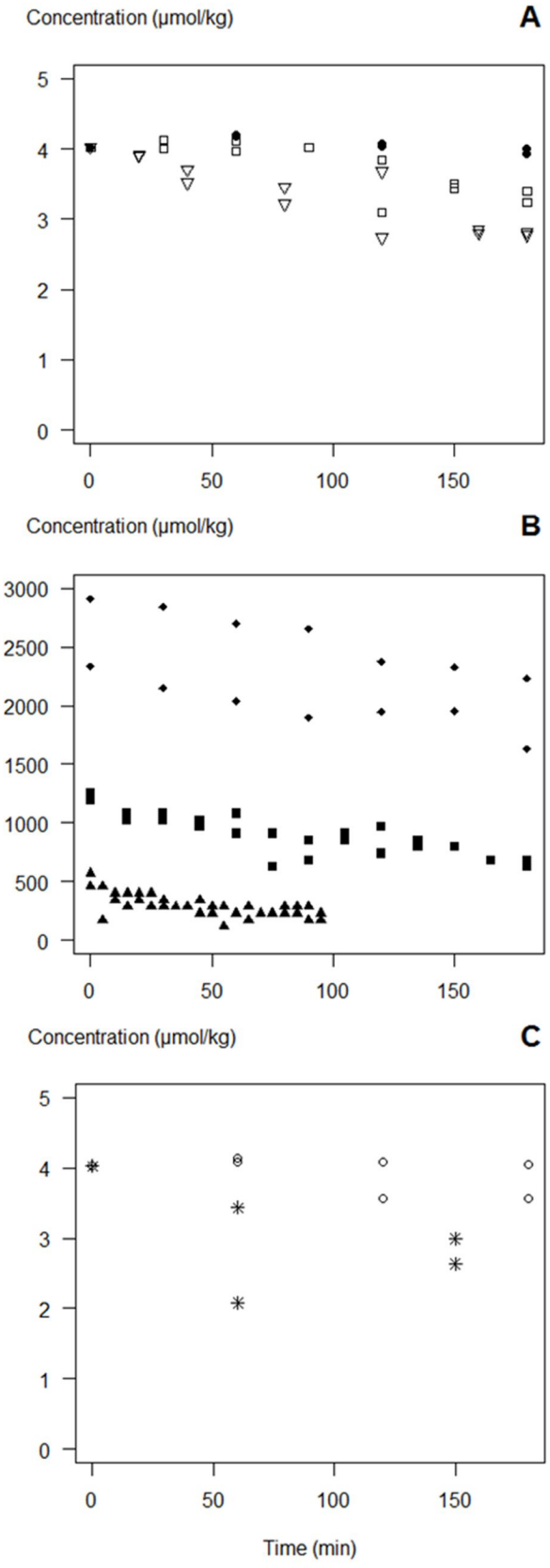

Figure 42: Impact of the concentration of ascorbic acid on the stability of 5-CH_3-H_4folate in apple and carrot purée heated at 80°C.

A: Stability of 5-CH_3-H_4folate in apple purée containing an initial ascorbic acid concentration of ▽ 570 µmol/kg, □ 1420 µmol/kg or ● 2840 µmol/kg.

B: Ascorbic acid content in apple purée during heat treatment as a function of the initial concentration.

Ascorbic acid initial concentration: ▲570 µmol/kg vs. ■ 1420 µmol/kg and ◆ 2840 µmol/kg.

C: Stability of 5-CH_3-H_4folate in carrot purée after an ascorbic acid addition of ✳570 µmol/kg or ○2840 µmol/kg.

Oey et al. (2006) reported that already low amounts of ascorbic acid (284 µmol/L) are sufficient to completely protect 5-methyltetrahydrofolate (4 µmol/L) at 80°C. From these results it can be concluded, that besides the amount of ascorbic acid present in the medium, another, time-dependent factor seemed to crucially influence the stability of 5-methyltetrahydrofolate. Dissolved oxygen is consumed within one hour in apple purée which is heated at 80°C (Chapter II) and degradation of 5-methyltetrahydrofolate does not proceed when the medium is deprived of oxygen before the heat treatment (Delchier, Ringling, Cuvelier, et al., 2014). In this study, an implication of reactive oxygen species, which might have been formed when oxygen was still present, seemed probable as degradation still occurred after one hour. Boatright (2016) measured up to 32.5 µmol/L of hydrogen peroxide during the oxidation of 100 µmol/L ascorbic acid. The formation of hydrogen peroxide and other reactive oxygen species during the oxidation of ascorbic acid and possibly also other components in the apple purée medium, might explain the time-limited, protective effect of ascorbic acid. Longer protection with increasing amounts of ascorbic acid is consistent with the assumption of reactive oxygen species being involved, since the probability rises that reactive oxygen species encounter an ascorbic acid before a 5-methyltetrahydrofolate molecule.

Another explanation might also be an implication of sugar derivatives. In model solution, ascorbic acid protects 5-methyltetrahydrofolate in the presence of fructose (100°C, 45 minutes) by reducing 5-methyldihydrofolate, the first oxidation product (Figure 40) (Verlinde et al., 2010). In apple purée, the protective effect might however be counteracted by an accumulation of sugar degradation products in the course of time and might explain the shifted degradation initiation of 5-methyltetrahydrofolate. In addition, since some ascorbic acid degradation products, namely dehydroascorbic acid and 2,3-diketogulonic acid, are also dicarbonyls which are even more reactive Maillard precursors than sugars, they might participate to the shifted degradation beginning (Ortwerth & Olesen, 1988a; Reihl et al., 2004; Roig et al., 1999; Slight et al., 1990).

As the composition of the food matrix may potentially influence the stability of 5-methyltetrahydrofolate, the latter was subsequently supplemented to carrot purée to verify if stability changes. As in apple purée, when no ascorbic acid was added, 5-methyltetrahydrofolate was very

fragile and degraded rapidly. Its stability in carrot purée in the presence of 570 µmol/kg and 2840 µmol/kg of ascorbic acid was not different to that in apple purée when the same amount of ascorbic acid was added (Figure 42 C). Stability was attained for the presence of 2840 µmol/kg of ascorbic acid but not when only 570 µmol/kg were used for supplementation. Coherently, Mnkeni and Beveridge (1983) who studied the stability of fortified folic acid in apple and tomato juice (100-140°C), did not observe any difference between degradation paces in the two studied food media despite of their different composition. In accordance to results obtained for apple purée and to those of Indrawati et al. (2004) who worked with carrot juice (50 mg/100 g ascorbic acid) that was heated at 120°C for 40 minutes, the presence of ascorbic acid in carrot purée was linked to high stability of 5-methyltetrahydrofolate.

The degradation accelerating impact of fructose is concentration independent (Verlinde et al., 2010) and therefore can supposed to be similar in apple and carrot purée as both contain fructose. The polyphenols epigallocatechin-gallate and catechin are reported to enhance the stability of 5-methyltetrahydrofolate (Rozoy et al., 2013). Polyphenols in the investigated apple purée had either a negligible protective effect as no difference compared to carrot purée was observed, or the protection extent of carotenoids, which are also antioxidants, and polyphenols was equal. However, effective protection of 5-methyltetrahydrofolate in apple and carrot purée was only attained when vitamin C was added.

4.4 Temperature effect (60-80°C)

Temperature diminishes the stability of 5-methyltetrahydrofolate in model solution according to the Arrhenius equation (Indrawati et al., 2004; Nguyen et al., 2003; Oey et al., 2006). However, the extent depends on the used pH and buffer ions (Indrawati et al., 2004; Paine-Wilson & Chen, 1979). It was hypothesized that stability for 3 h could be reached in apple purée with only 570 µmol/kg of ascorbic acid by decreasing temperature. Dropping temperature from 80°C to 70°C, or 60°C did not increase its stability though (Figure 43). Supply of energy was apparently not the limiting factor for degradation in this temperature range. Indrawati et al. (2004) studied the impact of temperature on the stability of 5-

methyltetrahydrofolate in orange juice, kiwi purée, carrot juice and asparagus. In the mentioned study, orange juice and kiwi purée contained 50 mg/100 g of ascorbic acid equaling 2840 µmol/kg, and carrot juice and asparagus amounted to 10 mg/100 g ascorbic acid corresponding to 570 µmol/kg. Consistently to the results of this study, they observed no marked impact between the amount that degraded at 70°C or 80°C in the different food matrices.

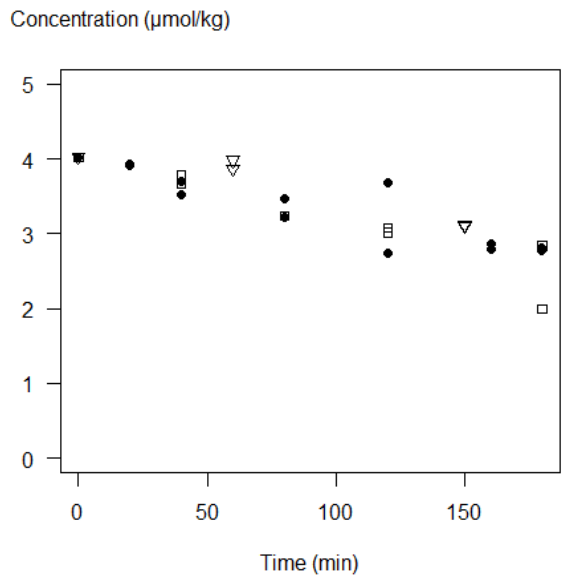

Figure 43: Impact of temperature on the degradation of 5-CH_3-H_4folate.

5-CH_3-H_4folate in apple purée containing 570 µmol/kg ascorbic acid

during a heat treatment at ▽ 60, □ 70 and ● 80°C.

5. Conclusion

The heat susceptibility of 5-methyltetrahydrofolate was confirmed in apple and carrot purée. Polyphenols and carotenoids that could, due to their anti-oxidative properties, potentially enhance the stability of 5-methyltetrahydrofolate are far from being as effective as the addition of vitamin C. Stability can only be obtained in the presence of vitamin C. The vitamin C amount which is necessary to entirely protect 5-methyltetrahydrofolate depends on the intended duration of the heat treatment. The time length of complete stability can be prolonged by increasing the vitamin C concentration. The molar vitamin C concentration is not directly related to the concentration of 5-methyltetrahydrofolate.

Even if the amount of ascorbic acid is still in excess to the amount of 5-methyltetrahydrofolate or dissolved oxygen, degradation starts in the course of time. A time dependent factor seems to intervene. A temperature decrease in the range 60-80°C does not have a marked impact on the stability of 5-methyltetrahydrofolate. Thus, by heating purées at 80°C, the risk of microbial growth can be decreased, while the same nutritional value is maintained as when heated at 60°C.

Chapter IV

This chapter is planned to be published. Authors were Anna-Lena Herbig, Célia Mousties and Catherine M. G. C. Renard. It is entitled "Impact of three warming up methods on the stability of vitamin C and 5-methyltetrahydrofolate in apple and carrot purée".

1. Abstract

Two methods that are used at people's home to warm-up food namely a microwave and an Actifry ® device, and a system that is usually employed in canteen kitchens to keep food warm, were examined on their impact on the stability of vitamin C and 5-methyltetrahydrofolate. Purées were heated until ebullition with the microwave, which lasted 1.5 minutes. Purées which were warmed-up by the Actifry ® device needed around 20 minutes to attain 80°C. When purées were kept warm by a water bath, the vitamin stability was pursued for 180 minutes at 80°C. During the latter, stability was examined at the surface and bottom of recipients that were filled with 600 g or 200 g of purée corresponding to a filling height of 5 cm and 1.5 cm respectively.

Although vitamin C and 5-methyltetrahydrofolate are usually referred to as heat- and oxygen-susceptible molecules, none of the methods led to major vitamin losses. In terms of the warm holding method, no difference was observed between withdrawals at the surface and the bottom of vessels for neither of the two filling levels. Vitamins were also fairly stable at the surface of recipients where oxygen exposition is high.

Keywords: Reheating, warm holding, ascorbic acid, folates, supplementation

2. Introduction

Vitamin C and vitamin B_9, the latter is also known as folate(s), are essential micronutrients which have to be ingested as they cannot be synthetized by humans' metabolism. Vitamin supplementation can be used to increase their intake. Folic acid, a synthetic folate vitamer, is predominantly used for enrichment of folates however it might mask vitamin B_{12} deficiency. Thus, the natural abundant vitamer, 5-methyltetrahydrofolate, has been proposed as alternative for supplementations (Pietrzik et

al., 2010b; Scaglione & Panzavolta, 2014). It is susceptible to deterioration though. To comply with the intention of enrichment, that is to supply a certain vitamin amount, the stability study of vitamin C and 5-methlytetrahydrofolate is indispensable.

Vitamin C and 5-methyltetrahydrofolate are both sensitive to oxygen and temperature (Delchier, Ringling, Cuvelier, et al., 2014; Dhuique-Mayer et al., 2007). When oxygen is absent, no degradation of vitamin C and 5-methyltetrahydrofolate occurs (Chapter II; Delchier et al., 2014). Up to 100°C, oxygen is still soluble and in dynamic equilibrium with headspace oxygen (Van Bree et al., 2012). Dissolved oxygen in supplemented apple and carrot purée is consumed entirely within one hour at 80°C in contrast to model solutions (Chapter II). After the depletion, oxygen is only available in the headspace. The filling height and thus oxygen availability is the factor that influences predominantly the stability of vitamin C in in apple purée serum that is heated at 80°C in Eppendorf tubes (Chapter I).

Unfortunately, 5-methyltetrahydrofolate is very unstable in contrast to folic acid, and degrades rapidly in model solution (Delchier, Ringling, Cuvelier, et al., 2014; Paine-Wilson & Chen, 1979) as well as in food matrices (Chapter III). By adding ascorbic acid, complete stability of 5-methyltetrahydrofolate can be attained during heat treatments (Oey et al., 2006; Chapter III). However, the amount of ascorbic acid which is necessary for total protection cannot be generalized, for two reasons. First, even if ascorbic acid is available in molar excess compared to the amount of dissolved oxygen or 5-methyltetrahydrofolate, degradation starts after an initial protection period (Chapter III). And second, the amount can be supposed to depend on the overall oxygen availability which is influenced by the experimental set-up. The exposition to headspace oxygen, which is determined by the geometry of the recipient and increases when the medium is stirred, can be supposed to play a crucial role. Nevertheless, the time length of protection of 5-methyltetrahydrofolate can be extended by increasing the vitamin C amount (Chapter III).

Reheating of food exhibits other constraints than cooking in terms of temperature and time length. Temperature recommendations for hot keeping of food are very similar in different European countries. with a minimum temperature of 63°C in France (Direction de l'information légale et administrative, 2011) and 65°C in Germany (Bundesinstitut für Risikobewertung, 2008). The aim of

this recommendation is to impede growth of spore-forming bacteria. In terms of length, it has to be distinguished between people's homes and canteen kitchens. At the latter, food is usually held warm up to several hours.

The impact of warming-up methods on the stability of vitamin C and 5-methyltetrahydrofolate has rarely been studied. Data exist for microwave heat treatments. Depending on the heating length and the type of food, vitamins are preserved or lost. For example, vitamin C in strawberry purée was lost to 2 % when treated at 90°C for 10 s and to 12 % when heated at 120°C for 10 s under the conditions of Marszalek et al. (2015). It was not lost in kiwi purée after a treatment at 1000 W for 340 s (Benlloch-Tinoco et al., 2014) and to 46 % in broccoli after a treatment at 1000 W for 5 minutes (Vallejo et al., 2002). Bureau et al. (2015) observed that vitamin C and folates in 13 frozen vegetables were well retained after a microwave treatment between 10-18 minutes without addition of water. Johansson et al. (2008) compared the impact of microwave heating (900 W/5.5 minutes), reheating in a saucepan until food reached 85°C and warming-up in an oven (225°C/40 minutes) on folates stability in 10 precooked vegetarian food products. All methods significantly influenced the stability of folates but variability of each method was high and thus no recommendation could be inferred.

At people's homes, microwaves are often employed. Warming-up with an Actifry ® device, which is usually used to cook French fries with a reduced amount of oil, could be an alternative. The Actifry ® device is equipped with a scoop which automatically stirs the food product. Heating is carried out via conduction and additionally by ventilation of hot air in the headspace. Each heating method has its own, critical parameter for vitamin losses. When food is warmed-up by a microwave, heating until ebullition is necessary to ensure food safety. When using an Actifry ® device, the product is heated at a lower temperature but is stirred and is thus more exposed to headspace oxygen. In canteen kitchens, duration can be a critical factor as food is usually kept warm up to several hours.

The objective of the present work consisted in assessing the stability of vitamin C and 5-methyltetrahydrofolate after a warming-up treatment carried out by three different systems. It was envisaged to optimize the heating protocols in case that vitamins are lost. Apple and carrot purée were therefore supplemented with the two vitamins on a pilot scale. The impact of a microwave and an

Actifry ® device, and that of a warm holding method which is usually used in canteen kitchens, was studied. When studying the impact of the latter method, special attention was paid on the effect of filling height of recipients and degradation at the bottom and surface of vessels.

3. Material and Methods

3.1 Production and supplementation of apple and carrot purée

Supplemented apple and carrot purée (25 kg respectively) were produced by the "Centre Technique de la Conservation des Produits Agricoles (CTCPA)" in Avignon/France. In Figure 44, production steps are shown.

For the preparation of apple purée, apples of the type "Golden" were washed and cut into pieces. Afterwards, the apple pieces were chopped at 80°C for one hour in a "Stephan" (Stephan, Hameln, Germany) which is a combination of a mixer and a cooker. The apple mix was passed through a "Parmentière" (Eillert, Ulft, Netherlands) that is a filter, to discard apple cores and seeds. An amount of 25 kg of the purée was weighed, let cool down for 30 minutes and subsequently homogenized for 10 minutes in the "Stephan". For the production of enriched carrot purée, the same procedure as for apple purée was applied except that the washing and cutting step, and filtration by the "Parmentière" were omitted since deep-frozen carrot pieces were used.

Afterwards, ascorbic acid and 5-methyltetrahydrofolate were supplemented. Therefore, ascorbic acid (12.625 g) and 5-methyltetrahydrofolate (50.5 mg) were dissolved in 250 mL ultrapure water. The aimed ascorbic acid concentration in the purée was 50 mg/100 g and that of 5-methyltetrahydrofolate was 0.2 mg/100 g. The solution with the two vitamins was poured in the purée. Afterwards, the mixture was stirred for homogenization during 10 minutes in the "Stephan". The supplemented purée was transferred into cans which were deep-frozen (≤ -18°C) until experimentation.

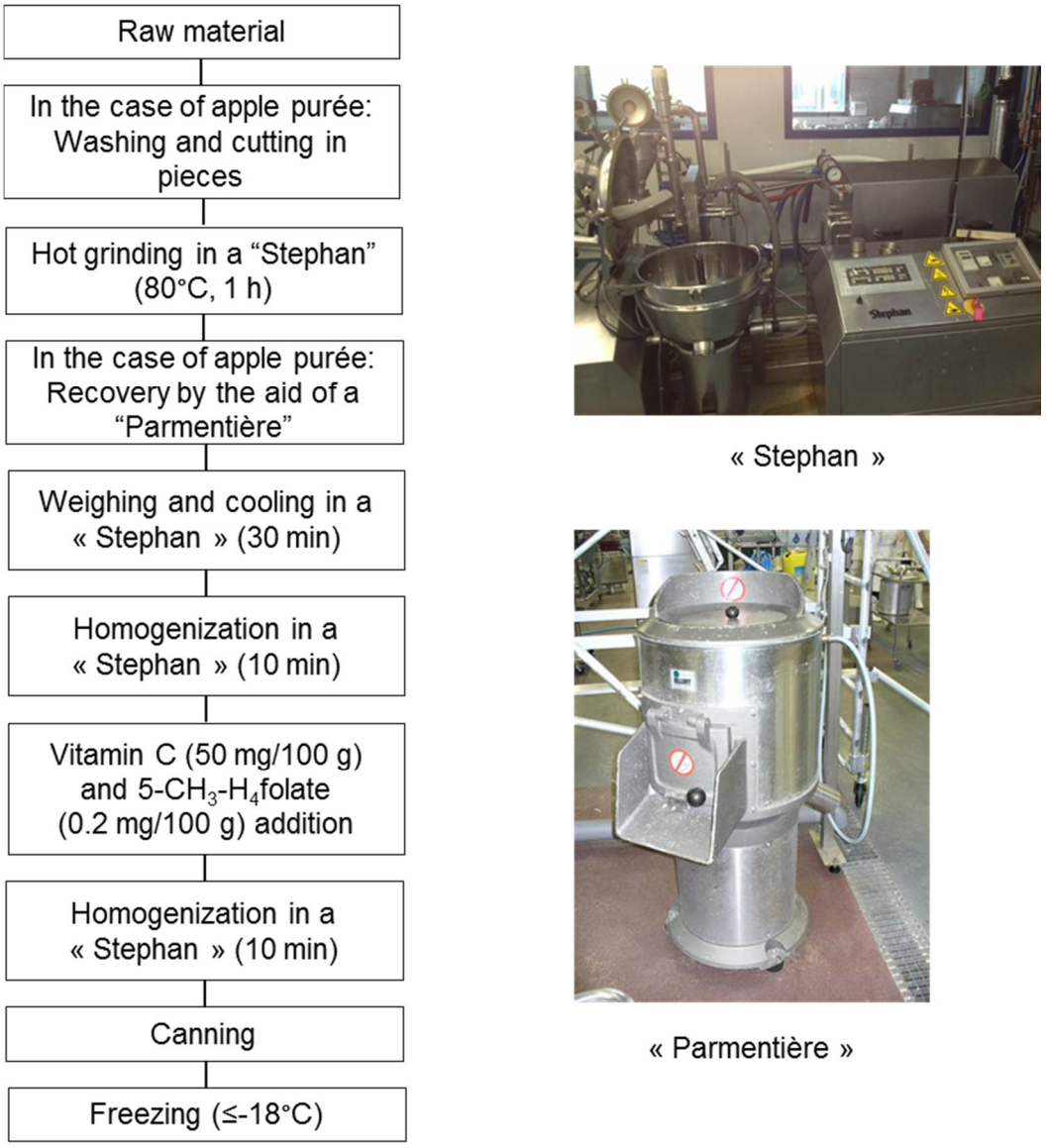

Figure 44: Processing steps of supplemented apple and carrot purée.

3.2 Reheating experiments

Purées were thawed overnight in a cold room (8°C) before each heat treatment.

3.2.1 Microwave warming-up

Thawed purée (250 g, corresponding to one can) was poured in a deep plate. A domestic microwave of the type Crisp FT439 Whirlpool (Whirlpool Corporation, Michigan, USA) was used for the heat treatment. The purée was heated until ebullition which took 1 minute and 30 seconds at 1300 W. One aliquot per plate of approximately 1 g was taken before and after the heat treatment and deep-frozen (≤ -18°C).

3.2.2 "Actifry ®" heating

The purée (1 kg) was weighed in the device. The integrated scoop of the "Actifry ®" device (Groupe SEB, Ecully, France) turned with a pace of 2 rounds per minute. Temperature in the Actifry ® was followed continuously and the treatment was stopped when 80°C were reached, which was achieved in about 20 minutes. Aliquots of around 1 g were withdrawn for vitamin analysis before and after the heat treatment and were immediately deep-frozen (≤ -18°C).

For determination of moisture contents, samples stayed for 4 days at 70°C in drying cabinets.

3.2.3 Warm holding method

A water bath of the type Royal Catering, RCBM 1/6 150 GN (Royal Catering Corporation, Chemnitz, Germany) was employed, which was endowed with six standardized 1/6 150 GN recipients (width x length x depth: 176 mm x 162 mm x 150 mm) and six cover lids with gaskets that hindered water evaporation. Each vessel corresponded to one time point. The purée was preheated up to 80°C in a pan before it was transferred to the six recipients. Temperature of the water bath device was fixed at 80°C. The first point of time curves corresponded to purée that was withdrawn after the preheating. During the heat treatment of 3 h, aliquots were regularly withdrawn at the surface and the bottom. The effect of the filling height was investigated by using 600 g or 200 g of purée for one vessel, which corresponded to a filling height of approximately 5 cm and 1.5 cm.

3.3 Ascorbic acid measurement

The method of Stevens et al. (2006) was employed which is based on a spectrophotometric detection. Aliquots of 500 mg were taken for analysis. A spectrophotometer (Safas Xenius, Monaco) was used for absorption measurements at 525 nm.

3.4 5-methyltetrahydrofolate analysis

The method of Ndaw et al. (2001) was applied. A HPLC connected to a fluorescence detector (RF-10AXL, Shimadzu Inc., Kyoto, Japan) was employed for analysis. The excitation wavelength was set at 295 nm and the emission wavelength at 356 nm. A guard column LiChrospher RP18 All Guard (7.5 x 4.6 mm, Alltech, Epernon, France) and a LiChrospher 100 RP18 column (250 x 4.5 mm, 5 µm, Alltech, Epernon, France) were used. The mobile phase consisted in acidified water (1 mL/L, v/v, formic acid) and acetonitrile. The gradient at the beginning was 5 % acetonitrile which was increased linearly to 58 % within 14 minutes and then to 100 % in 3 minutes. It was held for 11 minutes at 100 %, then decreased to 5 % acetonitrile in 2 minutes and was subsequently kept at 5 % for 5 minutes. The flow rate was 0.8 mL/min and the injection volume 25 µL. An external calibration was carried out. 5-Methyltetrahydrofolate was purchased at Schircks (Jona, Switzerland).

4. Results

4.1 Test of homogeneity

Homogenous distribution of supplemented vitamin C and 5-methyltetrahydrofolate within the apple and carrot purée batch was verified. Two aliquots, one at the surface and another at the bottom, of five respective cans were therefore withdrawn.

The vitamin C contents in apple and carrot purée, at the two locations of all cans gave almost identical values (Figure 45). The average concentration of all ten analyzed vitamin C contents, accounted to 51 ± 2 mg/100 g in apple purée and to 47 ± 2 mg/100 g in carrot purée. The measured concentrations

corresponded thus to the amount that was added. In addition, homogeneity in both matrices was indicated by low standard deviations.

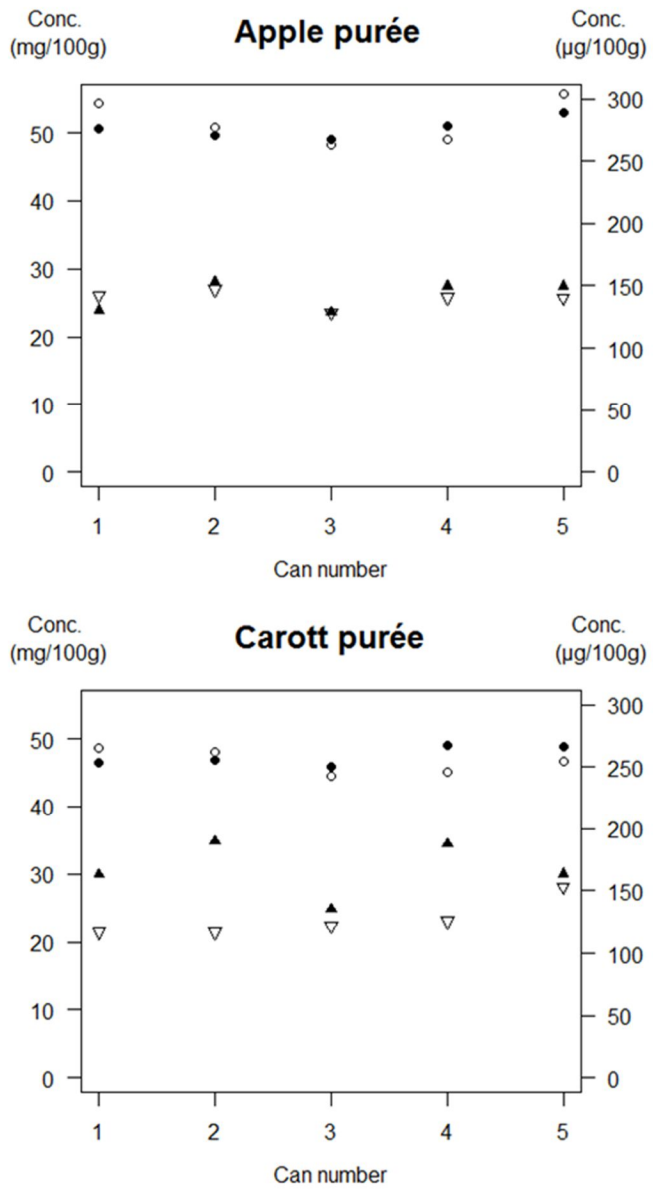

Figure 45: Test of vitamin homogeneity in apple purée and carrot purée.

Aliquots were taken at the surface and at the bottom of five respective cans.

Vitamin C content at the ○ surface and at the ● bottom.

5-CH_3-H_4folate content at the ▽surface and at the ▲bottom.

5-Methyltetrahydrofolate was, as vitamin C, equally distributed in apple purée (Figure 45). The mean value of all analyzed contents was 141 ± 9 µg/100 g. The determined amount was lower than the added content of 200 µg/100 g. Nevertheless, the low standard deviation indicated homogeneity of 5-methyltetrahydrofolate within apple purée. In carrot purée, contents at the surface of cans were slightly lower than at the bottom (Figure 45). The mean concentration of all contents withdrawn at the surface of cans was 127 ± 15 µg/100 g and that at the bottom was 168 ± 22 µg/100 g. The average concentration of all aliquots was 147 ± 28 µg/100 g. An accumulation of non-soluble particles in the surface region might have led to the lower 5-methyltetrahydrofolate concentration in carrot purée. The concentration discrepancy between the two locations was however not enormous and before starting heat treatment experiments, purées were homogenized again by the aid of a spoon to reduce this inhomogeneity.

4.2 Microwave warming-up

Supplemented apple and carrot purée were heated until ebullition which lasted 1.5 minutes at a power of 1300 W. After the heat treatment, the purée was homogenized by a spoon and samples were withdrawn. Vitamin C and 5-methyltetrahydrofolate withstood the treatment without major losses (

Table 10). High stability of vitamin C when food is heated by a microwave is in accordance with the result of Benlloch-Tinoco et al. (2014), Marszałek, Mitek, and Skąpska (2015) and Bureau et al. (2015).

Table 10: Vitamin C and 5-CH_3-H_4folate concentration before and after the microwave heat treatment.

Matrix		Time (min)	Vit. C (mg/100 g)	5-CH_3-H_4folate (µg/100 g)
Apple purée	Mean	0	52.1	229.2
	s.d.		*1.1*	*6.3*
	Mean	1.5	52.6	225.8
	s.d.		*0.4*	*5.7*
Carrot purée	Mean	0	46.0	210.2
	s.d.		*4.2*	*0.4*
	Mean	1.5	41.5	211.8
	s.d.		*0.3*	*4.1*

4.3 Actifry ® heating

The Actifry ® device heated apple purée slightly quicker than carrot purée. The aimed temperature of 80°C was attained in apple purée between 17 and 18 minutes whereas warming-up of carrot purée lasted between 19 and 21 minutes. Water was significantly lost during the treatment as evidenced by a weight loss of 18 ± 1 % in apple purée and 23 ± 3 % in carrot purée. This loss was determined by weighing the device before and after the treatment. However, it appeared to occur predominantly at the surface which was indicated by incrustation of purée only at the top. The water content inside the product stayed constant. In apple purée, the moisture content before the treatment accounted to 65.8 ± 15.8 % and afterwards to 72.1 ± 6.1 %, and in carrot purée to 86.9 ± 1.7 % before, and to 81.1 ± 6.2 % after the treatment. This result indicates that homogenization of purée during the heat treatment was weak. Under these conditions, as after the microwave heat treatment, minor to negligible degradation of both vitamins was determined (Table 11).

Table 11: Vitamin C and 5-CH_3-H_4folate concentration before and after the Actifry ® heat treatment.

Matrix		Time	Vit. C (mg/100 g)	5-CH_3-H_4folate (µg/100 g)
Apple purée	Mean	initially	44.0	245.8
	s.d.		*2.7*	*29.7*
	Mean	end	43.6	183.6
	s.d.		*1.0*	*63.3*
Carrot purée	Mean	initially	32.1	203.0
	s.d.		*2.9*	*41.2*
	Mean	end	33.2	232.2
	s.d.		*0.2*	*10.3*

4.4 Warm keeping method

After preheating the purée in a pan and transferring it to the vessel, the temperature in apple and carrot purée first decreased (Figure 46). The regulated temperature of 80°C was attained again after around one hour, but only at the bottom of vessels not at the surface. At the same position, no difference

between the temperature time curve in apple and carrot purée was observed (Figure 46). When recipients were filled with 600 g of purée, the mean temperature at the bottom between 60 and 180 minutes was 81 ± 1 °C in apple purée and 81 ± 2 °C in carrot purée. At the surface, the average temperature accounted to 64 ± 3 °C and to 64 ± 3°C respectively. When vessels were filled with 200 g, the same tendency was observed. The mean temperature at the bottom was 78 ± 1 °C in apple purée and 78 ± 4 °C in carrot purée, and at the surface 64 ± 5 °C and 68 ± 7 °C respectively.

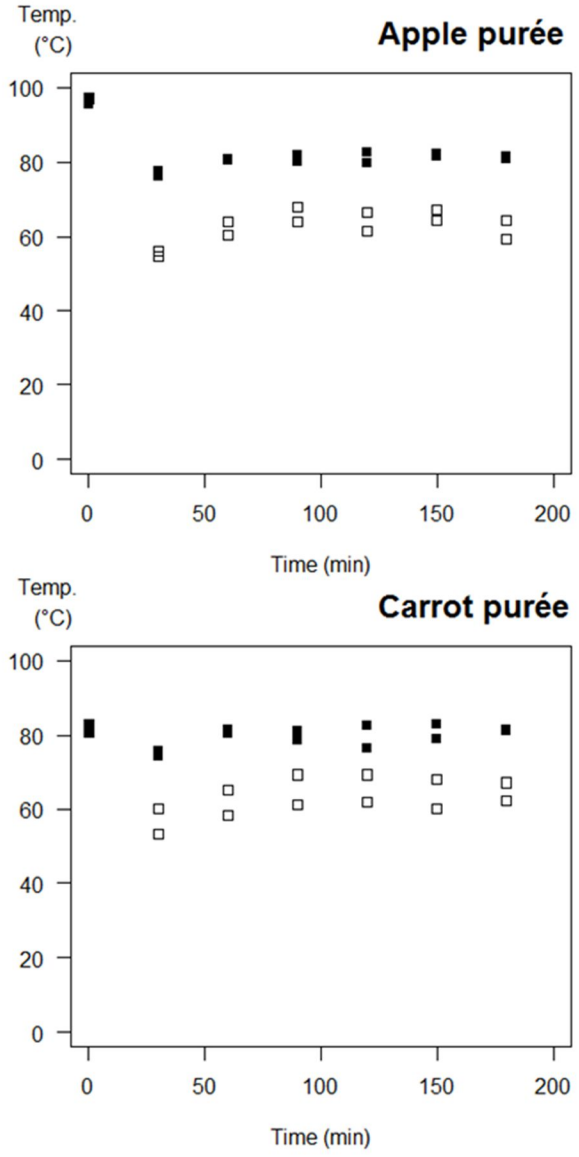

Figure 46: Temperature time courses during warm holding of apple and carrot purée.

Measured at the ■ bottom and at the □ surface of vessels that were filled with 600 g of purée.

Even if a temperature difference between bottom and surface positions of around 15°C existed, the respective temperature was, after the initial decrease, stable over time and also at the surface still in accordance to food safety regulations. Degradation rates neither of vitamin C nor of 5-methyltetrahydrofolate increase significantly with temperature between 60 and 80°C (Chapter I and III). It could thus be excluded that the temperature difference in the two locations influences degradation paces. It was however supposed that as a consequence of oxygen consumption in the medium and faster oxygen renewal near the surface by oxygen coming from the headspace, the degradation rate of vitamins at the surface is higher than that at the bottom. To verify this, vitamin C and 5-methyltetrahydrofolate contents were subsequently analyzed at the surface and the bottom of recipients filled with 600 g or 200 g of purée.

In apple purée, slight degradation of vitamin C was observed in the course of time. However, the degradation pace at the surface did not differ from the bottom of vessels (Figure 47). The filling volume of vessels did not impact the degradation either. The rate of degradation was identical when recipients were filled with 200 g or 600 g (Figure 47).

In carrot purée, initial values were already at the beginning much lower than the supplemented amount of 50 mg/100 g (Figure 47). This might be due to vitamin C degradation during storage since when homogeneity was tested, values still corresponded to the enriched amount. Although the initial content was lower than the supplemented amount, the heat treatment did not lead to vitamin C degradation (Figure 47). As for apple purée, no impact of the position where the aliquot was taken, or the filling height was established.

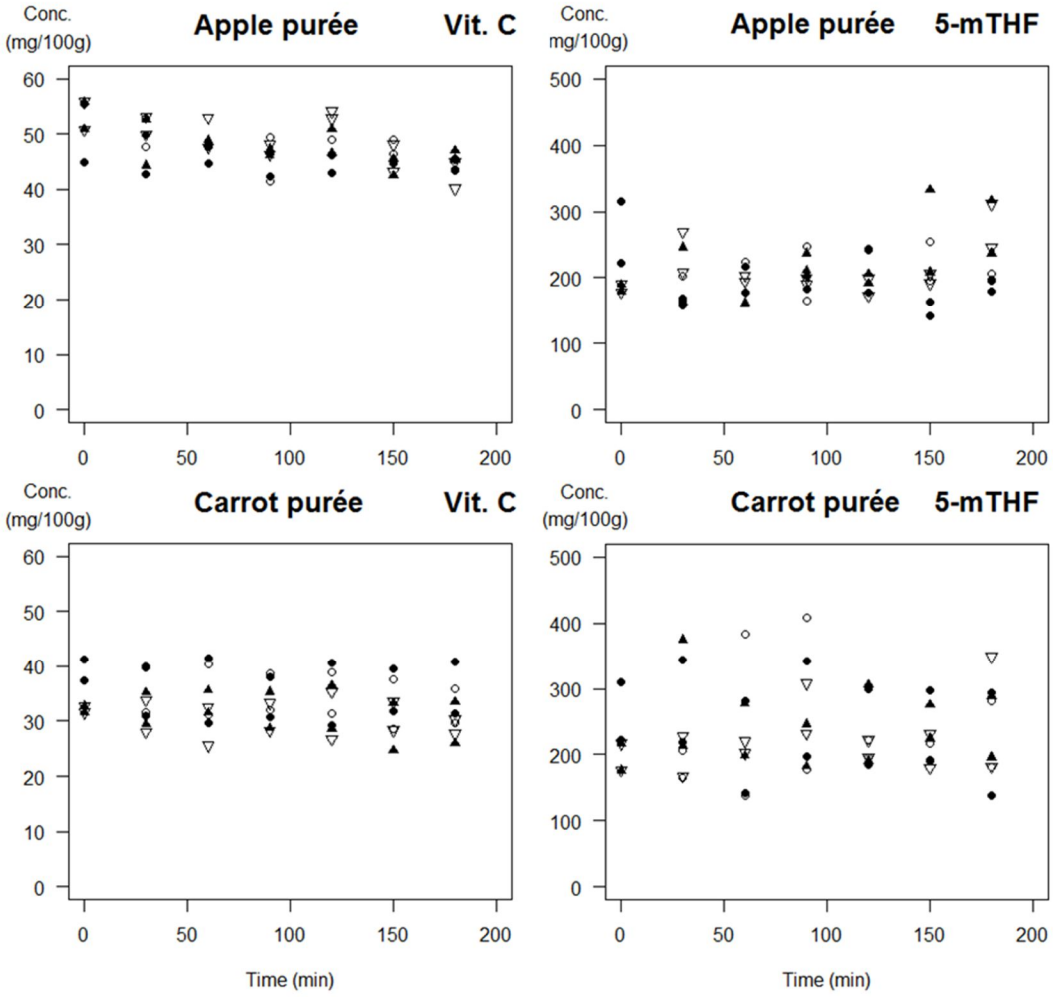

Figure 47: Concentration of vitamin C and 5-methyltetrahydrofolate (5-mTHF) during warm holding of apple purée and carrot purée.

Measured at the ○ surface and at the ● bottom of vessels that were filled with 600 g of purée, and at the ▽ surface and at the ▲ bottom of vessels that were filled with 200 g of purée.

Vitamin C degraded also on a lab-scale level slightly faster in apple than in carrot purée (Chapter I). No difference between degradation rates was determined when vessels were filled with 200 g or 600 g which corresponded to a filling height ratio of around 1:3. The impact of filling height which was ascertained on the lab-scale level (Chapter I) could not be confirmed on the scale-up level. In addition, stability was homogenous within the medium. Vitamin C degraded not faster near the surface, contrary to what has been observed for agar gel during storage at 20°C (Penicaud et al., 2011). An impact of the position where the aliquot is withdrawn might however also appear at elevated temperatures when degradation is in an advanced state.

Results concerning the stability of 5-methyltetrahydrofolate were less evident as a consequence of high standard deviations, especially in carrot purée (Figure 47). Already initial values varied quite strongly. Less disparity of values was observed when homogeneity within purées was tested or when the effect of a microwave or an Actifry ® heat treatment was examined. The variability of contents could not be ascribed to withdrawal at the surface or bottom position either, as values were randomly distributed (Figure 47). In addition, low variation of values was observed when the concentration of vitamin C in the course of time was analyzed. The elevated variability might thus result from the preheating phase which impacted only the concentration of 5-methyltetrahydrofolate

In apple purée, the average of all initial contents was 227 ± 62 µg/100 g and the mean of final amounts 236 ± 53 µg/100 g. In carrot purée, the mean accounted to 231 ± 57 µg/100 g and to 239 ± 74 µg/100 g respectively. Under these conditions, it appeared that 5-methyltetrahydrofolate was quite stable. This result is in accordance to lab-scale experiments which revealed that by adding 50 mg/100 g of vitamin C, 5-methyltetrahydrofolate is effectively protected during 3 h at 80°C (Chapter III).

5. Conclusion

Vitamin C and 5-methyltetrahydrofolate are commonly described as fragile molecules that are very sensitive to oxygen and temperature. The results of this study show however, that when they are

supplemented together to apple or carrot purée, both vitamins are fairly stable during warming-up of products. They were not or to a negligible amount degraded when heated by a microwave, an Actifry ® device, or when held warm for 3 . During the latter, stability was pursued at surface and bottom of the medium but no stability difference was ascertained. Even if exposition to headspace oxygen was high at the surface, this did not impact the vitamin stability. Modeling of vitamin losses or adaption of initial vitamin amounts, are thus not necessary. The vitamin amount that is enriched, is also maintained even if the product is reheated.

Conclusion and Perspectives

Vitamin C and folates are well known for their beneficial health effects. In the project OPTIFEL, elderlies' nutrition was intended to be ameliorated by supplementing fruit- and vegetable-based food products with important nutrients, among vitamin C and folates. As the two latter are usually referred to be easily degradable, studying their stability was deemed to be crucial to comply with the intention of fortification, which consists in increasing their absorption amount. The focus of this work was set on their stability during warming-up of food. It was aimed to find fundamental impacts of the degradation of vitamin C and 5-methyltetrahydrofolate at intermediate temperatures. In this temperature range, oxygen is still soluble, which raises questions concerning the degradation mechanism that is where and when oxygen is available. Another major interest consisted in understanding the stability in real food matrices to generate more applicable data as impacts may potentially be different than in model solution. In this chapter, main results and their importance plus new questions that emerged and may be worth studying in the future are described.

In the first study, it turned out that the stability of vitamin C is predominantly influenced by extrinsic factors such as temperature or filling height and not by intrinsic factors namely pH, concentration and food composition. Degradation kinetics were modeled the best by a zero order model that is the loss over time was constant and independent of the initial concentration. When the food product was changed, that is carrot purée (pH 5.5) instead of apple purée (pH 3.5) was used, no marked stability difference was observed. The stability in apple and carrot purée was very similar in spite of different composition. They can thus be interchangeably used for fortification. If the enriched concentration of ascorbic acid is wanted to be adapted, for example for needs of particular consumer groups, the amount that degrades per time will be the same making the control of degradation simple. The pH seemed not to influence stability in a food matrix.

In the temperature range which is of fundamental importance for warming-up of food (60-80°C) to follow microbial safety instructions, a temperature change had no impact on the stability. Hence, when products are heated at 80°C, the microbial safety marge is increased and at the same time the nutritional value of the product is kept as if heated at 60°C. Concurrently, this result raises new questions about the mechanism, in particular about the limiting factor, as the increase of energy supply

did not enhance the degradation rate anymore. The filling height of tubes was the factor which had the greatest influence on the stability of vitamin C on a lab-scale level. The causal origin, which was ascribed to oxygen availability as other factors remained the same, was thus investigated further in the subsequent study.

Up to now, no analytical method permits a quantitative overview of degradation products of ascorbic acid. Qualitatively, a broad spectrum has been identified in literature by applying various detection methods. It could thus be envisaged in the future to develop a detection method that allows quantitative detection, ideally of all degradation products, to gain more insight in the degradation mechanism of ascorbic acid and its interactions with other constituents of the food matrix.

Furthermore, proteins are supplemented to food products in the project OPTIFEL. As it is known from literature that strong interactions exist between degradation products of vitamin C, namely dehydroascorbic acid and 2,3-diketogulonic acid, and amino acids/proteins, the quantitative extent at intermediate temperatures could be worth studying as well as the impact of interactions on the nutritional value of the product.

To understand where oxygen is available at intermediate temperatures, in a closed system, in the course of time, the behavior of oxygen during heat treatments was studied in the second chapter. In a closed, water saturated system at 80°C, oxygen concentrations in the medium were equal to that in the headspace region when no oxidizable components were dissolved. In the presence of ascorbic acid, behavior of oxygen differed in model solution compared to real food products as apple or carrot purée. In model solution, oxygen was consumed slowly during the degradation of ascorbic acid and increased again after an initial depletion meaning that the consumption by ascorbic acid was slow and the renewal by oxygen coming from the headspace was faster. In apple and carrot purée in contrast, oxygen was depleted fast compared to model solutions implying that other oxidizable compounds contributed markedly to its consumption. It was not replaced to a measurable extent in the course of time and thus, oxygen was not available for vitamin C degradation anymore. It can be inferred that the oxidation mechanism of ascorbic acid in model solutions and real food products must differ as oxygen

is a necessary precondition its oxidation. The result highlights the importance of headspace oxygen for the degradation of ascorbic acid in real food matrices, in contrast to degradation in model solutions where dissolved oxygen stays abundant in the course of time. Diffusion of ascorbic acid to the surface of the medium and/or diffusion of oxygen from the headspace into the medium might be of crucial importance in food products during longer heat treatments. This result may also explain the impact of the geometry of recipients, the main result of the first study.

Furthermore, the presence of oxygen was not alone responsible for the degradation initiation of vitamin C since in ultrapure water, no degradation occurred after a heat treatment at 80°C for 8 h. Fe^{3+} ions in ultrapure water and constituents of the studied food matrices provoked degradation. However, they were only active under aerobic conditions. The concomitant presence of a trigger and oxygen seemed to be crucial for the degradation initiation. Furthermore, as triplet oxygen is a diradical due to two parallel spins in anti-bonding orbitals, it can only react with radicals (Choe & Min, 2005). It is therefore very likely, that oxygen and the trigger influenced the degradation by generating reactive oxygen species. Furthermore, it has been shown that hydrogen peroxide is generated during the oxidation of ascorbic acid and might thus lead to an auto-acceleration (Boatright, 2016). For profound comprehension of the oxidation mechanism in the course of time, studying the appearance, potential accumulation and transformation of reactive of oxygen species would be necessary. Therefore, the development of a detection method would be needed.

5-Methyltetrahydrofolate, a natural alternative for enrichments, was highly susceptible to deterioration also within food matrices like apple or carrot purée. Vitamin C was a very effective protector and entailed complete stability, which was however time limited. The duration of stability could be prolonged by increasing the amount of vitamin C. A temperature decrease in the range 60-80°C did not impact the stability.

To study why vitamin C became ineffective in the course of time, even if it was still in present in molar excess compared to the amount of 5-methyltetrahydrofolate or dissolved oxygen, the detection of reactive oxygen species and sugar derivatives might give valuable clues about the mechanism.

At a scale-up level, vitamin C and 5-methyltetrahydrofolate were fairly stable. No to minor losses were noticed in enriched apple and carrot purée which were heated by a microwave or an Actifry ®, or kept warm for 3 h at 80°C by a water bath. The influence of filling height could not be confirmed on this level. It seemed that due to the specific geometry (the surface-to-depth ratio was in both cases high) no effect was observed. For the future, it could be interesting to study another ratio that is a lower surface-to-depth ratio. It is possible that due to the bigger volume, the diffusion to headspace oxygen is less important as longer diffusion distances within the medium have to be covered. Measurement of diffusion rates of oxygen and ascorbic acid at intermediate temperatures could be helpful for interpretations.

For the OPTIFEL project, high stability of vitamin C and 5-methyltetrahydrofolate when supplemented together are good news. The amount that is fortified will not or only to a negligible amount be lost during warming-up of food products. This was not expected as they are commonly referred to be very fragile. No optimization of warming-up protocols has to be carried out and losses must not be modeled.

Mechanistic models are often used to explain degradation mechanisms. Many groups claim a pseudo-first order model for degradation of ascorbic acid which requires however that oxygen is available in excess. It has been pointed out by this work, that this is not the case in food matrices at elevated temperatures and thus for correct modeling, the concentration of oxygen must be incorporated in the mechanistic model.

Furthermore, it was highlighted by this work that stability studies in real food products are crucial as impacts factors and the availability of oxygen differ from model solutions. Studies in model solutions are helpful for understand the impact of individual components. However, factors that impact the stability of vitamins are numerous and interact with each. In addition also the experimental set-up has a fundamental impact. Hence, for predicting vitamin losses, the stability study under real conditions is important.

A generalization of the outcome of heat treatments is not possible which is indicated by diverging literature results in terms of models and loss yields. Also in this work, the set-up influenced enormously the stability. Vitamin C degraded on a lab-scale to an extent of approximately one third. On the scale-up level, in contrast, no degradation occurred. In addition, an impact of the filling height has been established on a lab-scale but not on a scale-up level. The commonly used mechanistic models, which are most of the time based on a first order model, are not sufficient to describe this phenomenon. The impact of oxygen and temperature seems to be complex and to depend on time, the experimental set-up and also on the composition of the medium. Dynamics of ascorbic acid and oxygen (namely diffusion and not convection processes in purées), the formation of reactive oxygen species and interactions with other components, might influence the degradation rate crucially.

Main findings of the thesis can be summarized as follows:

- The degradation pace of vitamin C depends on the experimental set-up which may be due to a crucial impact of the diffusion of ascorbic acid and oxygen. For degradation predictions, the vitamin stability under real conditions should always be studied.
- Impact factors in model solution differ from that in food matrices which can be supposed to be due to interactions with other constituents and different oxygen availabilities.
- Oxygen is abundant during heat treatments at 80°C in model solutions, but not in food matrices where depletion of oxygen occurs in the course of time without oxygen renewal. For correct mechanistic modeling, the concentration of oxygen must be included in the model.
- 5-Methyltetrahydrofolate can be effectively stabilized by ascorbic acid. The effect is however time dependent. The duration of complete stability can be increased by the amount of added ascorbic acid. It is however unclear why degradation is initiated in the course of time even if ascorbic acid is still present in excess.

For the future, it is important to go beyond the knowledge which was gained from mechanistic modeling. Therefore, studying dynamics of ascorbic acid and oxygen, appearance and potential

accumulation of reactive oxygen species and interactions with other constituents in the food product, would be interesting.

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Supplementary data

A B

Figure 48: Coloration after 8 h at 80°C of apple purée and apple purée containing ascorbic acid.

A: Apple purée

B: Apple purée containing ascorbic acid (3 mmol/kg)

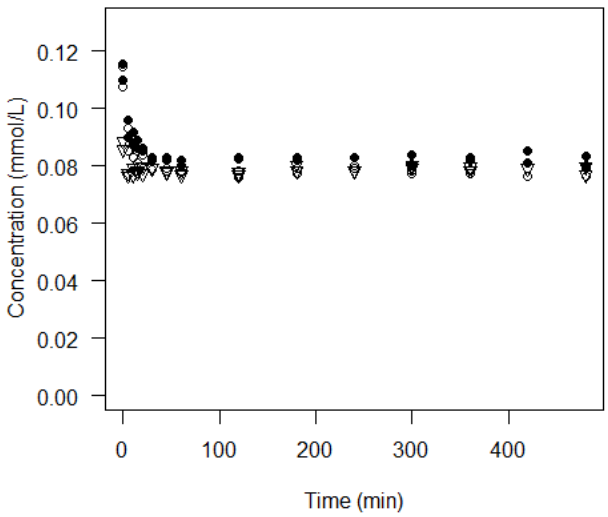

Figure 49: Oxygen time course at 80°C

in the ▽ headspace, and in the ○ surface and the ● bottom region

of water containing 20 µmol/kg Fe^{3+} ions.

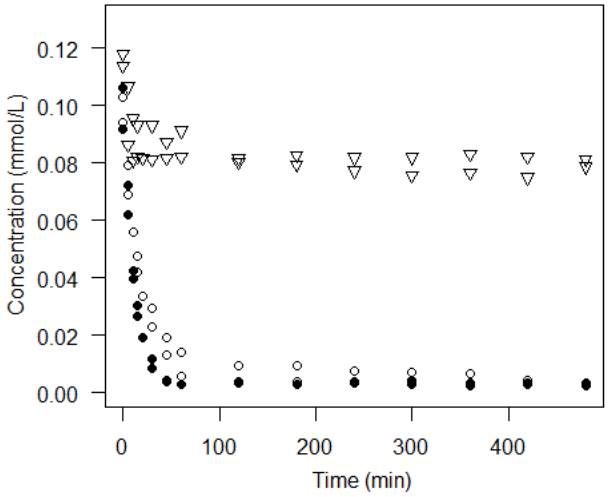

Figure 50: Oxygen time course at 80°C

in the ▽ headspace, and in the ○ surface and ●bottom region

of apple purée containing 3 mmol/kg ascorbic acid and 100 µmol/kg Fe^{3+} ions.

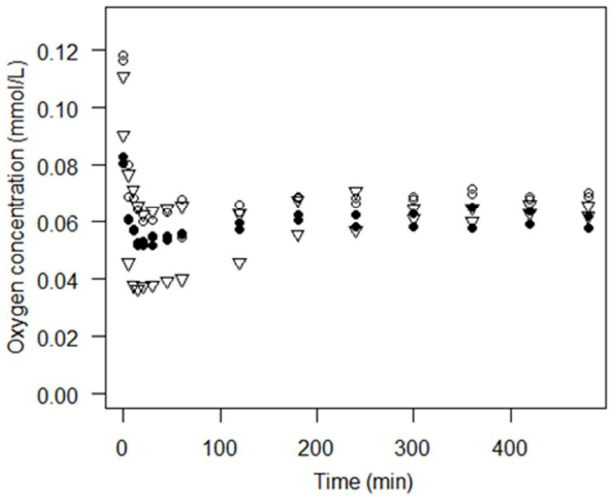

Figure 51: Oxygen time course at 80°C

in water containing 20 µmol/kg Fe^{3+} ions

and ●3, ○ 6 or ▽ 12 mmol/kg of ascorbic acid.

Measured at the bottom respectively.

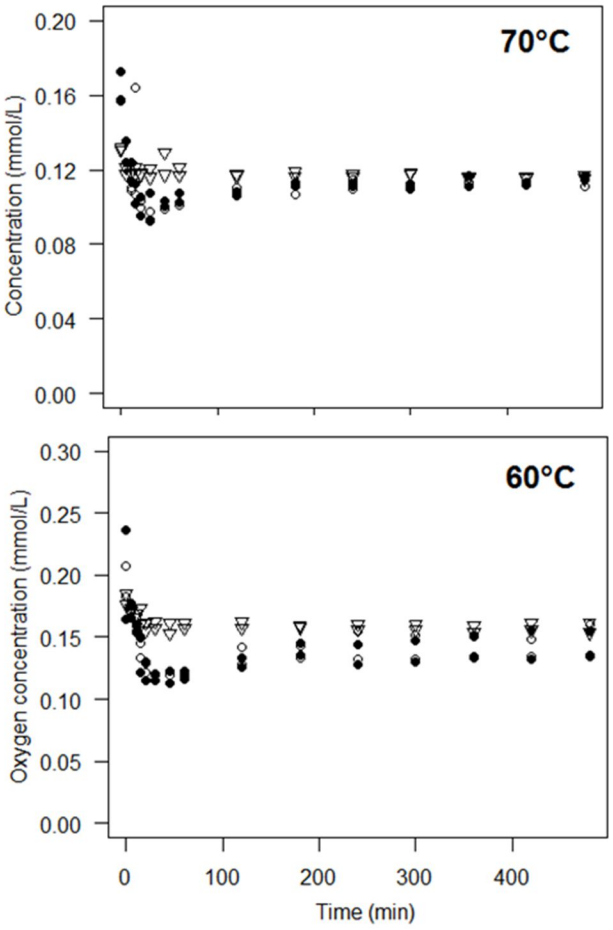

Figure 52: Temperature effect on oxygen contents

in water containing Fe^{3+} ions (20 µmol/kg) and ascorbic acid (3 mmol/kg)

Measured in the ▽ headspace, and in the ○ surface and ● the bottom region of the medium.

A: 70°C

B: 60°C

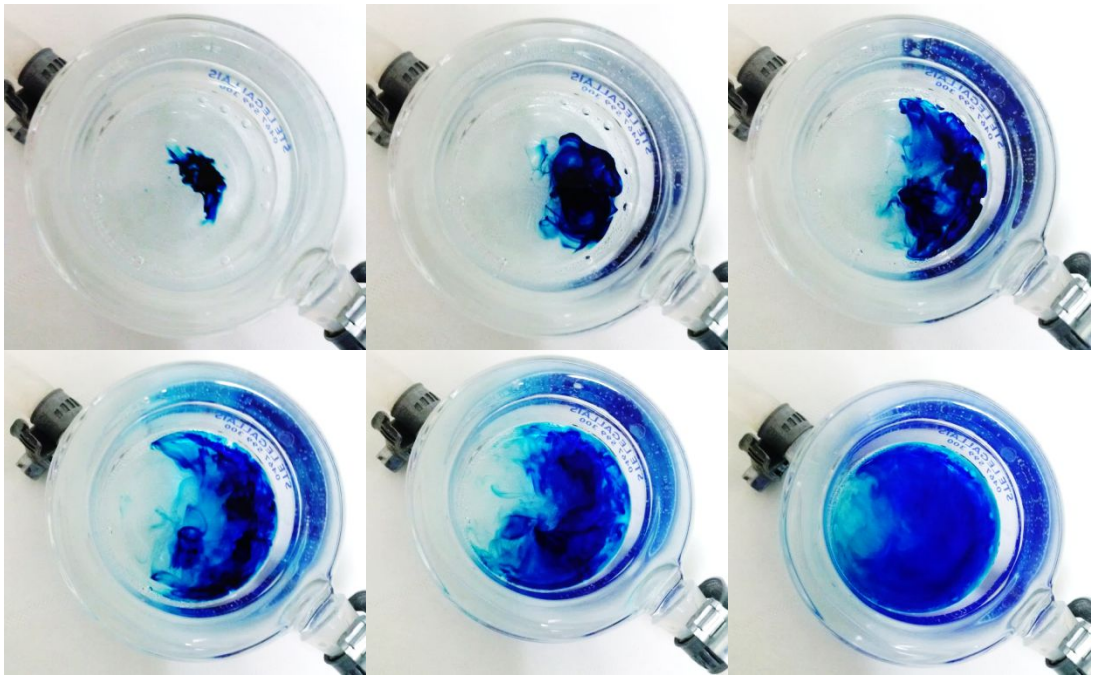

Figure 53: Methylene blue expansion in water at 80°C as a function of time.

Above left: 0 s, above middle: 2 s, above right: 4 s, bottom left: 6 s, bottom middle: 10 s; bottom right: 20 s.

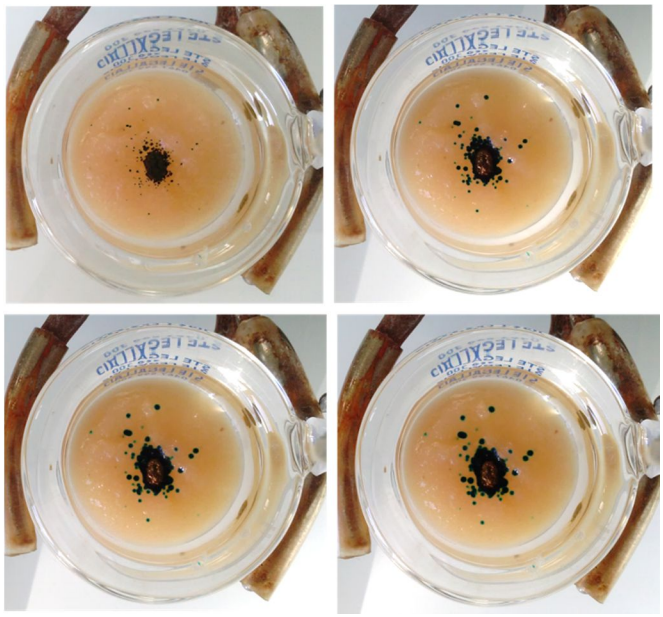

Figure 54: Methylene blue expansion in apple purée at 80°C as a function of time.

Above left: 0 s, above right: 30 s, bottom left: 60 s, bottom right: 90 s.

Table 12: Composition of apple and carrot purée.

Taken from the labels of commercial purées.

Nutritional value per 100 g	Apple purée (brand: POUCE)	Apple purée (brand: ALNATURA)	Carrot purée (brand: HIPP)
Energy (kJ)	270	231	126
Fat content (g)	< 0.5	0.4	0.1
Carbohydrates (g)	15.2	11.6	5.0
Proteins (g)	< 0.5	0.3	0.7
Salt (g)	< 0.01	< 0.01	0.1