

**FORENSIC CHEMICAL PROFILING OF TOMATO
SAUCES EXTRACTED FROM DIFFERENT SURFACES
USING HPLC-BASED CAROTENOID ANALYSIS**

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2026

CERTIFICATE

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DECLARATION

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ABSTRACT

Forensic trace analysis of residues from processed foods has received little attention, despite the fact that various studies have described tomato carotenoids for food quality and authenticity. This study aimed to compare carotenoid profile of tomato sauce residue extracted from different substrates. In this study, a representative tomato sauce residue was extracted from three surfaces, napkin, wrapping paper and cloth. Through a solvent extraction method, the extracted samples were extracted and subsequently analysed using high-performance liquid chromatography-photodiode array (HPLC-PDA). From the study, carotenoids were successfully extracted from tomato sauces residues on different substrates. Based on the HPLC profiles, seven identifiable peaks were evident. Statistical tests, including Shapiro-Wilk test, Kruskal-Wallis test, Pearson's correlation test and principal component analysis (PCA) were carried out, revealed that there was no significant difference in carotenoid composition across surface types. The overall findings demonstrate that carotenoid recovery from processed food residue was possible, supporting potential application of surface-based sampling in real-world contexts. In future work, more different common substrates should be included, along with varying environmental conditions that are unfavourable for sample preservation, to mirror real-world forensic cases.

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LIST OF ABBREVIATIONS

HPLC	High-Performance Liquid Chromatography
HPLC-DAD	High-Performance Liquid Chromatography with Diode Array Detector
RP-HPLC	Reverse-Phase High-Performance Liquid Chromatography
UHPLC	Ultra-High-Performance Liquid Chromatography
DAD	Diode Array Detector
PCA	Principal Component Analysis
UAE	Ultrasound-Assisted Extraction
UAEE	Ultrasound-Assisted Enzymatic Extraction
SFE	Supercritical Fluid Extraction
MAE	Microwave-Assisted Extraction
LC-MS/MS	Liquid Chromatography–Tandem Mass Spectrometry
FDA	Food and Drug Administration
SOP	Standard Operating Procedure
GGPP	Geranylgeranyl Diphosphate
IPP	Isopentenyl Pyrophosphate
DMAPP	Dimethylallyl Diphosphate
GGPS	Geranylgeranyl Pyrophosphate Synthase
PSY	Phytoene Synthase
PDS	Phytoene Desaturase
ZDS	ζ -Carotene Desaturase
LYC- β	Lycopene β -Cyclase
LYC- ϵ	Lycopene ϵ -Cyclase
CHY- β	β -Carotene Hydroxylase
CHY- ϵ	ϵ -Carotene Hydroxylase

ZEP	Zeaxanthin Epoxidase
VDE	Violaxanthin De-Epoxidase
NXS	Neoxanthin Synthase
EVOH	Ethylene Vinyl Alcohol
PVDC	Polyvinylidene Chloride
CO ₂	Carbon Dioxide
TPC	Total Phenolic Content
PDA	Photodiode Array
SPSS	Statistical Package for the Social Sciences
IBM SPSS	IBM Statistical Package for the Social Sciences

LIST OF SYMBOLS

α	Alpha (e.g., α -carotene, α -tocopherol)
β	Beta (e.g., β -carotene, β -cryptoxanthin)
γ	Gamma (e.g., γ -carotene)
ζ	Zeta (e.g., ζ -carotene)
μg	Microgram
g	Gram
$^{\circ}\text{C}$	Degree Celsius
nm	Nanometer
min	Minute
mL	Millilitre
μL	Microlitre
$\%$	Percentage
r	Pearson correlation coefficient
p	Probability (p-value)
df	Degrees of freedom
v/v	Volume per volume
$\geq$	Greater than or equal to
$<$	Less than
$\times$	Multiplication sign
$-$	Range indicator (e.g., 40–60 $^{\circ}\text{C}$)

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND OF STUDY

One crucial procedure in criminal investigations is the examination of trace material evidence, including food residues (Sumad-on, 2021). Food residues like tomato sauces can appear on a variety of substrates in forensic examinations, such as paper evidence, flooring materials, cutlery, and textiles. These remnants may be used as trace evidence to connect a particular incident to a suspect, item, or scene. However, accurate chemical identification can be hampered by the complexity of processed food matrices, substrate interactions, and environmental exposure. Apart from that, contamination and adulteration such as dilution with less expensive veggies, use of artificial colours or mislabelling of origin are ongoing problems in the food business (Hong et al., 2017). These issues, along with the complex food matrix nature of the tomato sauce, would make it more difficult to identify tomato sauce in crime scenes.

In relation to tomato, carotenoids, especially lycopene and β -carotene, are abundant in tomato sauces and give them their distinctive red hue (Tufail et al., 2024). These pigments may remain on surfaces even after drying or partial degradation since they are lipophilic and chemically stable substances (Borba et al., 2019). Carotenoids are promising chemical indicators for the forensic identification of residues derived from tomatoes due to their relative durability.

Evidence is not always found as a perfect sample in a forensic scene. Residues can be discovered on a variety of surfaces, including textiles, cookware, clothing, and packaging. A complex food matrix can be selectively adsorbed or degraded chemical components when it interacts with a surface material such as plastic, glass, cloth, or ceramic, leading

to changes in the detectable profile. Therefore, a crucial step in validating the analytical approach for practical forensic applications, like verifying the presence of a particular sauce at a scene or locating the source of a stain, involves understanding how the carotenoid profile of a tomato sauce transfers to and persists on various surfaces.

For the study of carotenoids, high performance liquid chromatography (HPLC) provides excellent sensitivity and selectivity. It might be feasible to create repeatable fingerprints that represent the origin of the sauce as well as the effect of the substrate by comparing the chromatographic profiles of tomato sauces recovered from different substrates. The purpose of this work is to create and verify a carotenoid profiling in the tomato sauce samples.

1.2 PROBLEM STATEMENTS

Forensic trace analysis of residues from processed foods has received little attention, despite the fact that several studies have described tomato carotenoids for food quality and authenticity considerations. Tomato sauces may stick to a variety of surfaces in real forensic situations, such as paper, metal, glass, cloth, or wood, which may change the chromatographic reaction and extraction effectiveness.

The effects of typical domestic and commercial surfaces on the recoverable carotenoid profile of tomato sauces have not been thoroughly studied. A distorted chemical fingerprint could occur from differential adsorption, oxidation, or degradation of certain carotenoids on these surfaces, which could provide false-negative matches or incorrect source classification.

There are substantial gaps in the application of HPLC for forensic profiling from residue samples on different surfaces, despite the method's proven use for carotenoid detection in regulated food samples. Standard HPLC techniques are best suited for pureed or homogenous liquid food samples. The effective extraction, recovery, and analysis of carotenoids from complicated, dried, and adherent residues on less-than-ideal substrates lack reliable and proven procedures.

Tomato sauce residues from various substrates cannot yet be profiled or compared using a standardised analytical procedure. Therefore, creating a repeatable process for HPLC-based profiling and carotenoid extraction can improve the forensic analysis of food traces and facilitate evidence comparability in criminal investigations. technique for tomato sauces recovered from various substrates using HPLC.

1.3 RESEARCH HYPOTHESIS

Based on the identified problems and research objectives, this study was guided by the following testable hypothesis. Concerning the fundamental interaction between the sample and substrate, it was hypothesised that the surface material critically impacts the analytical result. Specifically:

- Alternative hypothesis: There is a significant difference in the HPLC carotenoid profile recovered from a representative tomato sauce sample when dried on napkin, wrapping paper and cotton cloth.

Null hypothesis: There is no significant difference in the HPLC carotenoid profile recovered from a representative tomato sauce sample when dried on napkin, wrapping paper and cotton cloth.

1.4 RESEARCH OBJECTIVES

The study aims to investigate the profile of tomato sauce residues extracted from different substrates through a HPLC-based carotenoid profiling method for forensic application. Specifically, the objectives are:

- To develop the extraction of carotenoids from tomato sauces on various substrates, such as napkins, wrapping paper and cotton cloth.
- To determine carotenoid composition in tomato sauce samples using HPLC-DAD
- To compare carotenoid profiles among tomato sauce residues extracted from the different substrates.

1.5 SIGNIFICANCE OF STUDY

This study is important for applied food science, analytical chemistry, and forensic science.

Chemical analysis forensic procedures currently in use are primarily designed for pure and large-scale samples. This study proposes a proven procedure for the systematic recovery and characterisation of food residues from real-world substrates such as clothing, utensils and packaging, bridging the gap between controlled laboratory analysis and the complicated reality of crime scene evidence as it aims to reliably extract and analyse the carotenoid signatures from different surfaces.

From a basic scientific standpoint, this work offers vital and methodically collected information on a variable in analytical chemistry that is frequently disregarded, which is

the effect of the substrate on analyte recovery and profile integrity. The literature lacks a controlled study on how porous and non-porous surfaces selectively adsorb, degrade, or retain carotenoids, even though HPLC carotenoid analysis of tomato related products is well-documented. This work advances the disciplines of analytical science and food chemistry by measuring the surface-induced changes to the chromatographic profile.

In practical application, it seeks to produce a repeatable, reliable, and comprehensive HPLC-DAD technique that is especially tailored for the forensic examination of carotenoid-based food residues. The study gives forensic laboratories a workable and adaptable solution by offering a clear standard operating procedure. This strengthens the overall scientific rigor of forensic chemistry practice by lowering the requirement for internal method development for such samples, improving the dependability and admissibility of generated evidence in court, and encouraging consistency in analysis across several laboratories.

CHAPTER 2

LITERATURE REVIEW

2.1 CAROTENOIDS

The light-absorbing chromophore that gives carotenoids their vivid colours with a conjugated polyene chain in the structure. Carotenoids are tetraterpenoid hydrocarbons made up of eight carbons, isoprene units. Numerous significant physiological characteristics, including antioxidant activity, are granted upon carotenoids by their structures. As a result, they are crucial in preventing lipid peroxidation in cells and organisms (Typ & Ym, 1999).

Carotenoids are found in nature as both cis and trans isomers, albeit they are mostly found in the more stable all-trans isomeric form (Gupta et al., 2015). Carotenoids have different absorption spectra that typically display three closely spaced peaks (Britton et al., 1994). In the visible spectrum, most carotenoids show a large absorption band with a shoulder and two to three maximum absorption wavelengths. Cis-isomers exhibit absorption in the UV region (330–350 nm), producing a cis-peak and a hypochromic shift in the spectrum. Carotenoid isomers can be identified by comparing the absorbance of the main absorption peak and cis peak, which differ based on the isomers (Hsu et al., 2024).

2.1.1 CHEMISTRY OF CAROTENOIDS

Carotenes and xanthophylls are the two categories of carotenoids (González-Peña et al., 2023). Carotenes, including lycopene, α -carotene, β -carotene, and γ -carotene, are insoluble in polar solvents and extremely soluble in organic solvents (García-Barrientos et al., 2013). On the other hand, xanthophylls can be dissolved in both organic and polar

solvents, such as ether and hexane (García-Barrientos et al., 2013). They are carotenes that have been oxidized to produce acids, alcohols, aldehydes, and ketones.

The chloroplasts are where carotenoids are biosynthesised (González-Peña et al., 2023). From review article by González-Peña et al. (2023), geranylgeranyl pyrophosphate synthase (GGPS) catalyses the production of two molecules of geranylgeranyl diphosphate (GGPP) from isopentenyl pyrophosphate (IPP) and dimethylallyl diphosphate (DMAPP). Phytoene synthase (PSY) then condenses two molecules of GGPP into phytoene. ζ -carotene desaturase (ZDS) and phytoene desaturase (PDS) then desaturate phytoene into lycopene. Lycopene- ϵ (LYC- ϵ) and β -cyclase (LYC- β) catalyse the cyclisation of lycopene into α -carotene (α pathway) and β -carotene (β pathway), respectively.

Carotenes are used to make xanthophylls; α -carotene ring- ϵ hydroxylase (CHY- ϵ) forms lutein through the α pathway, while β -carotene hydroxylase (CHY- β) catalyses the conversion of β -carotene into β -cryptoxanthin through the β pathway. Zeaxanthin epoxidase (ZEP) can transform zeaxanthin into violaxanthin, while violaxanthin de-epoxidase (VDE) may transform violaxanthin into zeaxanthin. Lastly, neoxanthin synthase (NXS) transforms violaxanthin into neoxanthin. The complete flow of carotenoid biosynthesis pathway as shown in Figure 2.1.

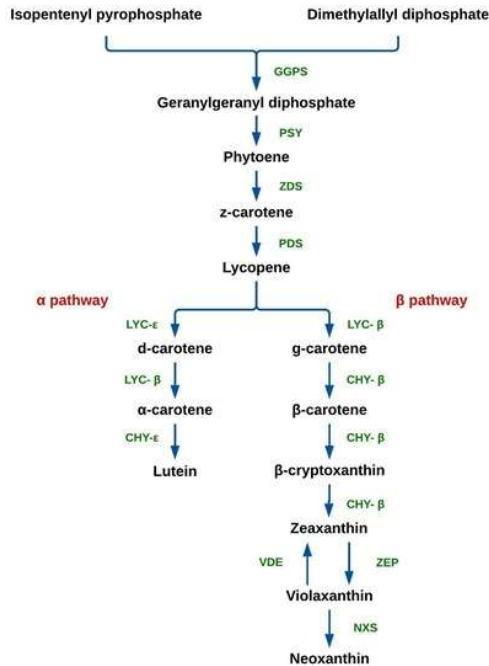


Figure 2.1: Carotenoid biosynthesis pathway in plants (González-Peña et al., 2023)

One of the most significant pigments that build up in fruits is carotenoids. Carotenoids are important accessory photosynthetic pigments that absorb light and guard against photooxidative damage (Lewinsohn et al., 2005). They also act as colouring agents in fruits and flowers (Lewinsohn et al., 2005). Lycopene is a carotenoid that acts as an intermediary in the manufacture of other carotenoids rather than accumulating in plant tissues (Hirschberg, 2001). However, substantial amounts of this pigment do build up in tomatoes and watermelons (about 30–100 µg/g fresh weight), giving the crops their characteristic red hue (Lewinsohn et al., 2005).

The carotenoid makeup (lycopene 95% and β-carotene ~5%) of common red watermelons is similar to that of the wild-type tomato, despite the fact that watermelon and tomato are botanically distinct (Tadmor et al., 2005). Research by Tadmor et al. (2005) has found that red watermelons may exhibit one of three carotenoid patterns: (1) high levels of lycopene and small amounts (less than 5% of the total carotenoids) of β-

carotene, similar to wild type tomatoes; (2) accumulation of mostly lycopene and significant (>10%) amounts of β -carotene, similar to the B fruit mutation of tomatoes; or (3) lycopene only, with no discernible amounts of β -carotene, similar to the fruit mutation of tomatoes. A recessive mutation may eliminate β -carotene and increase lycopene, and beta (B), a single dominant gene that raises β -carotene in the fruit (Ronen et al., 2000). Figure 2.2 shows the mutation and the respective biosynthetic pathway.

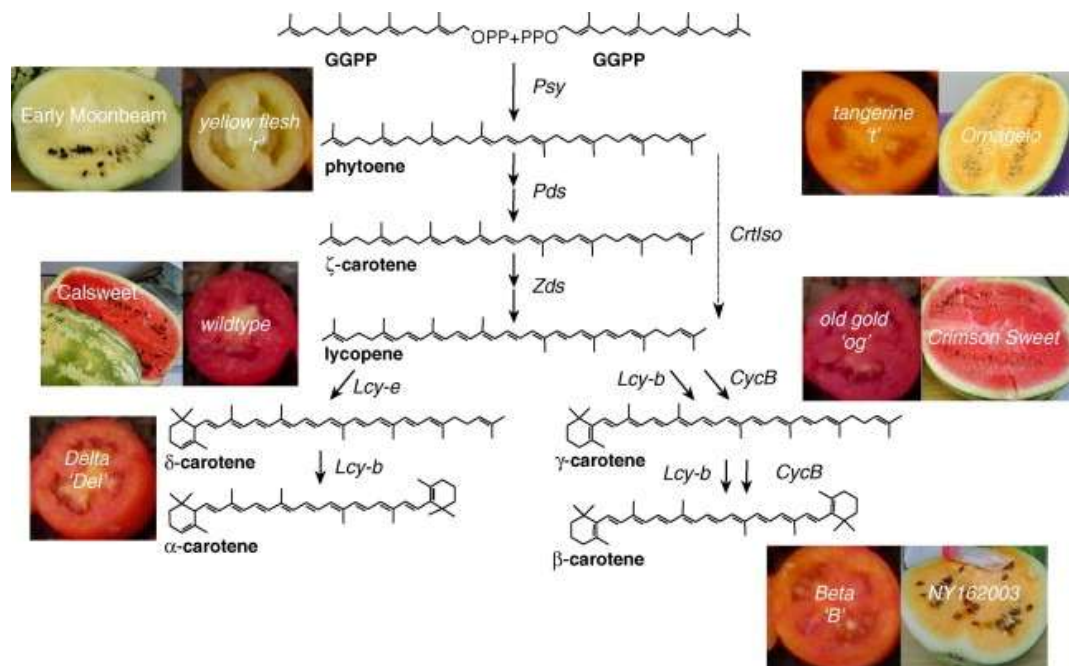


Figure 2.2: Tomato and watermelon fruit color mutants and their biosynthetic pathways (Hirshberg, 2001)

2.1.2 CAROTENOIDS DEGRADATION

When free radicals like singlet molecular oxygen and other reactive species attack the structures, they disintegrate. The carotenoid molecules' isomerisation, oxidation, and fragmentation are the typical degradation processes. The trans-form of carotenoids isomerises to the cis form when exposed to heat, light, and acids. Conversely, oxidation is caused by light, enzymes, prooxidant metals, and co-oxidation with unsaturated lipids.

Low molecular weight molecules are expelled during pyrolysis, which takes place at extreme heat (Typ & Ym, 1999).

Fresh tomato fruits mostly contain lycopene in the all-trans configuration (Shi & Maguer, 2000). Isomerisation and oxidation are the primary processes that lead to tomato lycopene degradation during processing (Shi & Maguer, 2000). Due to extra energy input, isomerisation transforms all-trans isomers into cis-isomers, creating an unstable and energy-rich station. An indicator of the possible health advantages of tomato-based foods would be the degree of lycopene isomerisation during processing (Shi & Maguer, 2000). In tomato-based foods, thermal processing (freezing, retorting, and bleaching) typically results in some lycopene loss (Shi & Maguer, 2000). The all the trans forms are isomerised to cis forms by heat. Temperature and processing time cause the cis-isomers to rise. Dehydrated and powdered tomatoes often have low lycopene stability unless they are prepared correctly and stored in an inert, hermetically sealed environment (Shi & Maguer, 2000).

2.2 TOMATO FRUITS

The tomato (*Lycopersicon esculentum* L.) is the second most important vegetable in the world, both in terms of quality and quantity, behind potatoes (Motamedzadegan & Tabarestani, 2018). The Mexican Nahuatl Indian word *tomati* is where the word "tomato" originates. It developed from the cherry tomato (*L. esculentum* var. *cerasiforme*) in the South American Andes (Jy et al., 2004). The tomato is a warm-season crop that is a member of the Solanaceae family of fruit vegetables (Motamedzadegan & Tabarestani, 2018). For best growth, it needs daytime temperatures of 25–30 °C and nighttime temperatures of 16–20 °C (Garg and Cheema 2011). Fruit sets best between 18 and 24 °C, with nighttime temperatures being more important than daytime ones. According to

Benton-Jones (2008), the tomato fruit is categorised as a berry and can range in size from small cherry varieties with only two ovary divisions (locules) to commercial cultivars for the fresh market with four to six locules.

2.2.1 CAROTENOIDS IN TOMATO

Tomatoes are a rich source of carotenoids (Orsi et al., 2021). Lycopene, α -carotene, β -carotene, γ -carotene, ξ -carotene, cyclolycopene, neurosporene, lutein, violaxanthin, neoxanthin, zeaxanthin, α -cryptoxanthin, and β -cryptoxanthin are among the more than 20 carotenoids that have been previously identified in tomatoes and tomato-based products (Shi et al., 2004; Guil-Guerrero and Reboloso-Fuentes, 2009). Tomato fruits have an uneven distribution of carotenoid content, and the cultivar (genotype), level of maturity, climate, environmental factors, and cultural techniques all have a significant impact on its composition (Martí et al., 2016).

Tomato plants produce carotenoids in their leaves, flowers, and fruits. The leaves contain large amounts of lutein, which serves as a photoreceptor during photosynthesis. Flowers are rich in xanthophylls, violaxanthin, and neoxanthin, which give them their distinctive yellow hue. Lycopene, the primary carotenoid at this stage of maturation, gives the ripe fruits of *Lycopersicum esculentum* its striking red hue (Martí et al., 2016). Ripe tomato fruits contain lycopene (about 90%), β -carotene (around 5–10%), and lutein (less than 1%). The distinctive colour of mature tomatoes is mostly caused by lycopene and β -carotene (Kotíková et al., 2009). These carotenoids are crucial elements in routine analyses that determine the fresh tomatoes' quality attributes (Kotíková et al., 2009).

2.2.2 TOMATO SAUCE AND ITS PROCESSING AFFECTS ON CAROTENOID

The FDA defines catsup, ketchup, or catch-up as food made from one or any combination of the following ingredients: (1) the liquid from mature red or red-dish tomatoes; (2) the liquid from the residue from preparing such tomatoes for canning, consisting of peeling and cores with or without such tomatoes or pieces thereof; and (3) the liquid from the residue from partial extraction of juice from such tomatoes (FDA, 1977).

Products' naturally inherent carotenoid makeup is changed during food preparation. The processing steps result in reactions caused by heat, acids, light, or oxygen exposure (Schieber & Weber, 2016). Total carotenoid content and antioxidant capacity have increased by 30% and 15%, respectively, as a result of thermal treatment. The enzymes ϵ - and β -carotene cyclase, which are involved in the synthesis of β - and α -carotene, may be activated during tomato processing. Thus, increasing the synthesis of β - and α -carotene (Vallverdú-Queralt et al., 2015).

The nutrients in fresh tomatoes as well as the consequences of processing and storage have an impact on the nutritional content of processed tomato products (Motamedzadegan & Tabarestani, 2018). Food processing generally results in the loss of specific molecules, such vitamins, which lowers the nutritional value of staples (Motamedzadegan & Tabarestani, 2018). The quantity of lycopene found in both processed and raw tomato products is displayed in Figure 2.3.

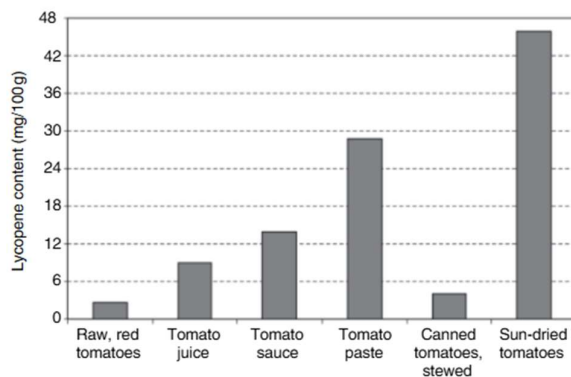


Figure 2.3: Effect of processing on lycopene content of selected tomato products (USDA, 2016)

Research by Klopotek et al. (2005) focused on juice, nectar, wine, and puree made from strawberries. Samples were collected at various stages of production to assess the impacts of processing and investigate the antioxidant capacity as well as the concentrations of ascorbic acid, total phenolics, and total anthocyanins. Spectrophotometric analysis was used to determine the vitamin C level. This study demonstrated the decline of every parameter examined throughout the processing of strawberries into various products. Ascorbic acid content dropped as production time and processing stages increased, particularly during heat treatment.

Klopotek et al. (2005) found that fining turned out to be a gentle way to clear berry juices and wines without eliminating significant levels of total phenolics, according to research on total phenolics in strawberry products. Due to the polymerisation and condensation of monomer phenolics like anthocyanins, fermentation did not result in significant losses of total phenolics. When employing high temperatures, both the hydrophilic antioxidant capacity and total anthocyanins are reduced. During wine processing, anthocyanins also significantly dropped, mostly as a result of fermentation and pasteurisation.

Gärtner et al. (1997) had observed carotenoid concentrations in the chylomicron fraction to examine the lycopene bioavailability from a single dosage of fresh tomatoes or tomato paste (23 mg lycopene) consumed with 1.5 g corn oil. Both fresh tomatoes and tomato paste had the same lycopene isomer pattern. Following both treatments, there was no discernible difference in the triacylglycerol response in chylomicrons and therefore, tomato paste had a higher bioavailability of lycopene in humans than fresh tomatoes (Gärtner et al., 1997).

The purpose of study by Seybold et al. (2004) was to examine how various tomato processing methods affected the amounts of lycopene, β -carotene, and α -tocopherol. Following each stage of manufacturing, samples of tomato sauce, tomato soup, baked tomato slices, and tomato juice were collected at various heating times. Vitamin E and carotenoids were analysed using HPLC, with C30 columns. Lycopene, β -carotene, and α -tocopherol concentrations rose on a wet weight basis as a result of water loss during thermal processing. Depending on the provenance of the tomatoes utilized, lycopene concentrations varied on a dry weight basis, while β -carotene contents either dropped or remained rather consistent. Thermal processing caused β -carotene to isomerise, unlike lycopene. Short-term heating caused a considerable increase in the α -tocopherol levels.

In a study by Tonucci et al. (1995), various types of tomato-based thermally processed products were compared. It includes soups, whole tomatoes, catsup, spaghetti sauce, tomato paste, tomato puree, tomato juice, vegetable juice and tomato sauce. These samples were extracted and carotenoids were individually identified and quantified by reversed-phase HPLC. Lycopene, lycopene-5,6-diol, lutein, -carotenes, neurosporene, phytoene and phytofluene were among the carotenoids that were found and measured. As anticipated, lycopene was the most prevalent carotenoid, with concentrations ranging from 55 mg/100 g in tomato paste to 0.3 mg/100 g in vegetable beef soup. In tomato soup, the content of β -carotene was 0.23 mg/100 g, while in vegetable beef soup, it was 1.51 mg/100 g. All samples examined had very low levels of lutein (less than 0.2 mg/100 g), with the exception of tomato paste, which had 0.34 g/100 g. Out of all the tomato products, tomato paste had the greatest quantity of total carotenoids (87 mg/100 g). The loss of water from tomatoes during processing to create the paste was the cause of the increased concentration.

2.3 RECOVERY OF CAROTENOIDS

Bioactive compounds are extracted from photosynthetic pigments using a variety of methods, including traditional extraction procedures like maceration and/or Soxhlet extraction and cutting-edge extraction procedures like pulse electric field, ultrasound, supercritical fluid extraction, microwave, and hydrodynamic cavitation (Keisandokht et al. 2017). There are numerous drawbacks to the traditional extraction techniques. For instance, low extraction yield, extended extraction time, and excessive solvent use. Many recent studies in the literature have focused on novel extraction methods to get around these problems (Dastan et al. 2021).

2.3.1 ULTRASOUND ASSISTED EXTRACTION (UAE)

Compared to traditional extraction methods, ultrasound assisted extraction (UAE), one of these novel treatments, is straightforward and reasonably priced (Han et al., 2024). Sound waves having frequencies higher than the human hearing threshold (> 16 kHz) are referred to as ultrasound (Han et al., 2024). The temperature and pressure rise as a result of these sound waves, and the shock waves that come from the cavitation brought on by these variations in temperature and pressure account for the ultrasonic effect (Han et al., 2024). Ultrasound equipment with frequencies between 20 kHz and 10 MHz is employed in the food business (Han et al., 2024). The literature reported the use of UAE to extract lipids, proteins, carotenoids, hemicelluloses, and aromatic chemicals (Demirdoven et al. 2021).

Numerous studies have shown how effective UAE is at optimising bioactive molecule recovery under ideal circumstances. In contrast to traditional techniques, Demirdöven et al. (2021) found that UAE at 40°C for 40 minutes produced the maximum anthocyanin content in extracts from sour cherry pomace, with notable advantages in antioxidant

capacity, total phenolic content (TPC), and colour characteristics. In cases of Han et al. (2024), the UAEE or ultrasound assisted enzymatic extraction was found to be more successful than the enzymatic extraction based on the findings of physicochemical, color, and antioxidant activity analyses of carotenoid extracts.

2.3.2 SOLVENT EXTRACTION

In a comparative research Heffernan et al. (2016), solvent extraction (hexane/acetone, 7:3 at 50 °C for 24 h) produced the best yield of the xanthophyll-rich extract from brown macroalgae *Fucus serratus* when compared to supercritical fluid extraction (SFE) with carbon dioxide and SFE with carbon dioxide and ethanol as co-solvent. On the other hand, carotenoids could be extracted using SFE with carbon dioxide at 50 °C, 304 bar of pressure, and 60 minutes of extraction time, producing fucoxanthin of a greater purity. The comparison findings demonstrated that while SFE produced higher purity, solvent extraction produced the greatest overall yield of the target carotenoids. When compared to other solvent combinations, the use of a combination of polar (ethyl acetate) and non-polar (hexane) solvents produced the maximum output of carotenoids from tomato waste.

In research by Mezzomo et al. (2011), when compared to ultrasound-assisted extraction and Soxhlet extraction with hexane/isopropanol, it was found that maceration using acetone produced the best yield of total carotenoids from pink shrimp (*P. brasiliensis* and *P. paulensis*). The lack of a heating system during extraction, which prevents the thermal destruction of carotenoids and reduces the contact time between solvent and raw material, led the authors to conclude that maceration was better than alternative techniques. Because of the low viscosity and surface tension at boiling temperature, which had enhanced the diffusion and solubilisation of carotenoids, Soxhlet subsequently demonstrated a satisfactory yield of carotenoids.

Valduga et al. (2008) have examined various methods for extracting carotenoid pigments from *Sporidiobolus salmonicolor* CBS 2636. To break up the cell and release the intracellular carotenoids, several solvents such as dimethyl sulfoxide, petroleum ether, hexane, ethyl acetate, chloroform, and acetone, liquid nitrogen, and diatomaceous earth were utilised. The study's findings demonstrated that using several solvents had a synergistic effect on the amount of carotenoids recovered. The treatment that used liquid nitrogen and dimethylsulfoxide to disturb the cell produced the highest concentration of total carotenoids (913 µg/L), which was then extracted using an acetone/methanol (7:3, v/v) solution.

The type of food matrix and its characteristic carotenoid pattern should be taken into account while selecting the extraction solvent (Paradiso et al., 2020). The most used solvents for extracting carotenoids from food matrices are acetone and hexane (Saini & Keum, 2017). Most of the methods used in the literature on carotenoids in vegetables and microgreens were either acetone mixed with water (Samuolienė et al., 2017) or hexane (Kyriacou et al., 2018) and hexane/toluene (Xiao et al., 2012) combined with saponification.

2.3.3 SAMPLE PRE-TREATMENT

Considering carotenoids and extracting solvents are hydrophobic, the high water content of fruits and algal cells is widely thought to be detrimental to the effective extraction of carotenoids, especially with SFE (Durante et al., 2014). Durante et al. (2014) proved that food matrices appropriate for supercritical carbon dioxide extraction were successfully prepared using vacuum oven and freeze-drying pre-treatments. However, vacuum oven-

dried matrices exceeded oven-dried matrices in terms of carotenoid extraction yield. This could be because tissue and cell structure disaggregation increases the matrix's permeability to the fluid.

However, conventional thermal-based dehydration techniques, such as oven and microwave drying, could lead to carotenoids' isomerization and thermal degradation (Saini et al., 2014). Thus, food samples are desiccated using lyophilisation or freeze-drying to shield the carotenoids from deterioration caused by high temperatures.

2.4 HPLC ANALYSIS OF CAROTENOIDS

Thin layer chromatography, HPLC, and combinations of HPLC and mass spectrometry, such as matrix-assisted laser desorption ionisation-time of flight (MALDI-TOF), have all been used to find carotenoids in dietary samples (Britton et al., 1994). The most popular technique for identifying and measuring carotenoids combines UV-vis absorption detection with HPLC. Normal phase HPLC is not appropriate for carotenoid separation since it cannot effectively separate non-polar carotenoids (Britton et al., 1994). On the other hand, reverse phase HPLC makes it possible for the analyte and non-polar stationary phase to interact significantly more, which improves the resolution of carotenoids (Sander et al., 2000).

Sander et al. (2000) found that lutein and zeaxanthin were not separated using the monomeric C18 column, and nonpolar carotenoid isomers were weakly resolved. The polymeric C18 column allowed for better separation of the hydrocarbon carotenoids, but it did not resolve the cis/trans isomers of β -carotene. The polymeric C30 column was used to attain near baseline resolution of the components using the same mobile phase gradient. It was noted that stationary phase features have a significant impact on lycopene's

retention behaviour. Lycopene often elutes before α - and β -carotene in monomeric C18 columns, but it is strongly maintained and elutes after these carotenoids in polymeric C18 and C30 columns.

However, research by Khachik et al. (1997) found that for carotenoid separation, C18 columns with an isocratic or gradient mode were the best option. The authors also acknowledged that the C18 column ineffectively resolved positional isomers, especially lutein and zeaxanthin, and did not resolve geometrical isomers.

Sander et al. created a non-encapped reverse-phase high performance liquid chromatography (RP-HPLC) column containing triacontyl (C30) ligands to resolve carotenoids and their isomers to optimise chromatographic resolution and selectivity. Cis/trans-carotenoids could also be resolved by the polymeric C30 columns as proved by Rajendran et al. (2005). Carotenoids in a variety of matrices have recently been analysed using ultra-high performance liquid chromatography (UHPLC) technology and microwave-assisted extraction (MAE) was proven a more effective technique with reduced extraction time, higher total phenolic contents and greater antioxidant activities compared to conventional solvent extraction (Li et al., 2011). Higher peak capacities, narrower peak widths, increased sensitivity, and improved chromatographic resolution are among the benefits that UHPLC has over HPLC (Gupta et al., 2015). Mobile phase solvents are also significantly reduced due to the shortened analytical times (Gupta et al., 2015). Despite their low resolution of carotenoid isomers, C18 stationary phase columns have been utilised for carotenoid separation because C30 stationary phase columns are not commercially available for UHPLC (Gupta et al., 2015).

A study by Bijttebier et al. (2014) compared a C30 HPLC column with a C18 UHPLC column. Although using a C30 HPLC column improved the resolution of carotenoids, especially geometrical isomers, when compared to C18 UHPLC columns, the benefit was

offset by the increased run time required for the C30 HPLC column (100 min versus 23 min for the C18 UHPLC column). In a different work, Maurer et al. (2014) that resolved all major carotenoids found in broccoli (*Brassica oleracea* L. var. *italica*), carrot (*Daucus carota*), corn (*Zea mays*), and tomato (*Solanum lycopersicum*), showed that 11 main carotenoids could be separated in 13.50 minutes utilising a C18 column on UHPLC-UV for quicker analysis. However, they were only able to identify a small number of geometrical isomers using this technique. Even though the UHPLC techniques now in use allow for shorter run times, they are not very good at resolving *cis* isomers of various carotenoids.

CHAPTER 3

METHODOLOGY

This chapter describes the materials, analytical techniques, and experimental setup used to examine the effectiveness of carotenoids extraction from tomato sauce residues on various surface substrates, covering cotton cloth, paper napkin and polyethylene-coated wrapping paper. The methodology is designed to assess the effects of surface chemistry on carotenoid recovery and profile integrity. It combines a HPLC analytical technique created especially for carotenoid separation with a reliable extraction strategy modified from the previous literature by De Castro et al. (2021).

3.1 MATERIALS AND CHEMICALS

3.1.1 SURFACE MATERIALS

Three easily available substrates that are typical food-contact materials were chosen as follows:

- Cotton cloth (WOT Cotton Kitchen Towel 28cm, Malaysia)
- Commercial paper napkins (Scott® Kitchen Towel, White 2 Ply Kitchen Roll, USA)
- Food-grade polyethylene-coated wrapping paper (Cap Nenas, Paper Food Wrapping "D", Malaysia)

Before the experiment, all substrates were cut into size 5 x 5 cm.

3.1.2 TOMATO SAUCE SAMPLES

A representative commercial tomato sauce (Life Tomato Sauce, Region Food Industries Sdn. Bhd., Malaysia) was selected for consistency. The sauce was homogenised by

shaking it for two minutes before application on the substrate to ensure uniform carotenoid distribution.

3.1.3 CHEMICALS AND SOLVENTS

Extraction solvents used in this study are as follows:

- Acetone ($\geq 99.5\%$, Sigma-Aldrich, USA) – used for initial carotenoid extraction
- Petroleum ether (boiling range 40–60°C, Sigma-Aldrich, USA) – used for liquid-liquid partitioning

Drying agent used in this study was anhydrous sodium sulphate (Na_2SO_4 , $\geq 99\%$) (Labbox Chemie, Spain).

HPLC solvents used for the chromatographic separation are as follows:

- Acetone (HPLC grade, $\geq 99.9\%$, Fisher Scientific, UK) – used in mobile phase preparation
- Ammonium acetate (HPLC grade, 10 mM solution prepared in deionized water, Sigma-Aldrich, USA) – used as a buffer in the mobile phase

It was noted that the acetone was refrigerated at 4°C prior to use to enhance carotenoid stability during extraction procedure.

3.1.4 APPARATUS AND INSTRUMENTATION

The following apparatus and instruments were used in this study:

- Freeze dryer: ALPHA 1–4 LSCplus (Martin Christ, Germany)

- HPLC system: Shimadzu Prominence LC20A equipped with a photodiode array (PDA), autosampler, column thermostat, and a reverse-phase C18 column (Phenomenex Luna, 250 mm × 4.6 mm, 5 µm)
- Analytical balance: Mettler Toledo XS205 (Switzerland)
- Vacuum filtration setup with Büchner funnel
- Separating funnel (250 mL)

3.2 EXPERIMENTAL DESIGN

3.2.1 SURFACE CONTAMINATION AND DRYING PROTOCOL

A total 2 g of tomato sauce was weighed for each sample, with three replicates per surface type using a sterile spatula to ensure consistent coverage. The sauce was spread to cover approximately 70% of the surface. The prepared substrate squares were then packaged in a plastic container to undergo a freeze-drying process, using specifically the Model ALPHA1 (Martin Christ, Germany). Freeze-drying was continued until constant weight was achieved, indicating complete moisture removal which took about 96 hours.

3.2.2 CAROTENOID EXTRACTION PROCEDURE

The carotenoid extraction protocol was adapted from De Castro et al. (2021). The lyophilised or freeze-dried sample was scraped using spatula to be weighed. Then, the sample was macerated in mortar with 20 mL of refrigerated acetone and homogenised for one minute. The extract was then vacuum filtered through a Buchner funnel with filter paper. This step was repeated five times in total.

The mortar, residue, and funnel were washed with the cold acetone, into the conical vacuum flask. 20 mL of petroleum ether was placed into 250 mL separating funnel. The

extract was carefully transferred to the funnel, washing the conical vacuum flask with acetone and transferring the extract to the funnel. A volume of 20 mL distilled water was carefully added through the funnel walls to avoid the formation of an emulsion. The lower aqueous phase was discarded. The washing step was then repeated for nine more times, accumulating in total 200 mL of distilled water. The lower phase was discarded entirely in the last wash.

The upper phase was collected in a 25 mL volumetric flask covered with aluminium foil, passing the solution through a glass funnel containing 7.55 g of anhydrous sodium sulphate to remove residual water, using glass wool in the funnel holding the anhydrous sodium sulphate. The funnel was washed with the petroleum ether collected in the volumetric flask. The volume of the volumetric flask was filled up with petroleum ether until the mark. The solution was then transferred to 2 mL vial using micropipette and ready for analysis.

3.3 HPLC-DAD ANALYSIS OF CAROTENOIDS

The HPLC conditions as shown in Table 3.1 were adapted from Wills and Rangka (1996) method for carotenoid determination to achieve optimal separation of major tomato carotenoids and their possible isomers/degradation products.

Table 3.1: HPLC Parameters Used

Parameters	Condition used
HPLC system	SHIMADZU PROMINENCE LC20A HPLC system with a diode array detector (DAD)
Column	Reverse-phased Luna Phenomenex (250 x 4.6 mm)
Column oven temperature	35°C
Mobile phase	Solvent A: Methanol: Acetonitrile (1:1, v/v)