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Determination of Theobromine in Cocoa Products Using Liquid Chromatography: A Review

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ABSTRACT

This review aims to critically evaluate and compare published high-performance liquid chromatography (HPLC) methods for quantifying theobromine in cocoa-based products, with the aim of identifying a single analytical approach that is both practical for routine use in food manufacturing settings and readily transferable to quality control laboratories within cocoa production facilities. Theobromine is the principal alkaloid in cocoa beans and plays a critical role in the quality of cocoa-based products, influencing taste, aroma, texture, and health benefits. In addition to cocoa, theobromine is present in smaller amounts in green coffee beans, tea, and cola nuts, contributing to the characteristic bitter taste of chocolate and related products. HPLC is widely employed to determine organic compounds, including theobromine in cocoa products, due to its high resolution, reproducibility, and suitability for complex food matrices such as chocolate and cocoa powder. Compared to other techniques like capillary electrophoresis or gas chromatography, HPLC offers superior sensitivity for non-volatile and thermally labile compounds like theobromine, requires minimal derivatization, and accommodates aqueous mobile phases. Among the various detection methods coupled with HPLC, mass spectrometry is a highly feasible option, providing superior data precision and faster analysis, even for routine quality control laboratories. A common mobile phase consists of a mixture of water, methanol, and acetonitrile, acidified with phosphoric or formic acid. For industrial applications, a simple sample preparation method using an ultrasonic bath is ideal due to its ease of use and effectiveness. Additionally, heat treatment-based sample preparation provides a faster alternative to conventional, time-consuming extraction methods, making it highly suitable for routine quality control in the food manufacturing industry. This review covers literature published between 2001 and 2023. Following a thorough survey, nine articles were selected for detailed comparison.

Keywords: Theobromine, HPLC, Cocoa, Chocolate and Methylxanthines.

1. INTRODUCTION

1.1 Cocoa and Cocoa Products

Theobromine (3,7-dimethylxanthine) is the principal alkaloid of cocoa beans. It has the chemical formula $C_7H_8N_4O_2$ and is a derivative of purine, classified as a dimethylxanthine [1]. The scientific name of the cocoa plant is derived from the Greek origin Theobroma cacao, meaning "Food of the Gods." Cocoa has been documented since the Aztec and Mayan civilizations, particularly for its medicinal properties[2]. Theobromine is present in higher concentrations in cocoa. It contributes to the bitter taste of cocoa-based products, especially chocolate.

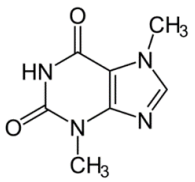

Figure 1. Molecular Structure of Theobromine

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Theobromine is a central nervous system stimulant belonging to the methylxanthine family. Although it is milder than caffeine due to its ability to bind to a smaller number of adenosine receptors, it has a longer half-life, meaning it remains in the body for an extended duration. Theobromine exhibits vasodilator properties, which can influence cardiovascular function, mood, and cognitive activities [3]. However, excessive consumption may lead to adverse effects such as heartburn, depression, anxiety disorders, and diuretic effects [4]. Importantly, theobromine is toxic to domestic animals such as dogs and cats, as their metabolism is significantly slower than that of humans [5].

This review focuses exclusively on the determination of theobromine in cocoa-based products, including cocoa beans, cocoa liquor, cocoa powder, cocoa butter, and finished chocolate products. Cocoa beans are the main raw material for chocolate and confectionery production. Cocoa liquor or cocoa mass is the direct ground product of cocoa beans, used to make high-quality chocolate and other confectionery items. By pressing cocoa liquor, cocoa butter, and cocoa powder are obtained, all of which are also used in chocolate manufacturing. Additionally, cocoa butter is frequently used in cosmetic and pharmaceutical applications.

On a dry basis, cocoa contains approximately 16% proteins, 50% lipids (cocoa butter), and 20% carbohydrates, in addition to alkaloids such as theobromine (0.8–1.4%) and caffeine (0.1–0.7%) [6]. Caffeine and theophylline are other methylxanthines found in cocoa alongside theobromine. Depending on the degree of fermentation and the type of cocoa bean, theobromine content varies from 1.5% to 3.0%, which has a significant impact on the quality of cocoa products [7]. From an industrial quality control perspective, theobromine content serves as a critical quality indicator during cocoa bean fermentation, directly influencing the taste, aroma, texture, and color of the final chocolate products. Regulatory and safety considerations further necessitate reliable theobromine quantification, particularly for developing pet-safe products and labeling functional foods.

1.2 Importance of Determining Theobromine

The food manufacturing industry requires a feasible, reliable, and efficient analytical method for theobromine determination in cocoa and cocoa-based products to address multiple needs. First, accurate quantification during fermentation would enable producers to identify optimal conditions, such as temperature, humidity, and duration, to achieve consistent product quality. Second, such a method would support the development of theobromine-enriched confectionery items positioned as functional health foods. Third, a robust analytical approach is essential for establishing safety thresholds in cocoa-based products, especially ensuring pet food manufacturers can accurately determine toxic levels of theobromine.

1.3 Limitations of Current Analytical Methods

Despite the widespread industrial use of cocoa in chocolate manufacturing, current analytical methods for theobromine determination present several specific limitations. Traditional techniques, including titrimetric procedures using silver nitrate, ultraviolet spectroscopy, thin-layer chromatography, ion chromatography, Fourier transform-Raman spectrometry, Fourier transform-infrared spectrophotometry, and gas chromatography [8], are either time-consuming, lack sensitivity, or require derivatization. Modern methods for the determination of theobromine in cocoa beans, cocoa, and chocolate products usually rely on HPLC. However, many published HPLC methods have been developed for research purposes [9] rather than routine industrial quality control, often employing time-consuming extraction procedures and expensive detection systems [5]. The chromatographic separation of theobromine from caffeine, a structurally similar methylxanthine co-present in cocoa, remains challenging, particularly with UV detection, as their maximum absorption wavelengths (theobromine at 272 nm, caffeine at 273 nm) [10,11] are nearly identical, leading to peak overlap and quantification errors.

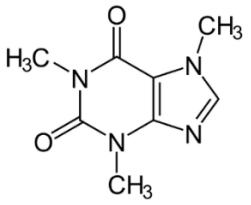

Figure 2. Molecular Structure of Caffeine.

Furthermore, while diode array detectors (DAD) and fluorescence detectors (FD) offer improved sensitivity, their high cost and compatibility issues with certain solvents and columns limit their practicality for routine industrial use. Existing studies rarely address simple, rapid, and cost-effective sample preparation methods that are essential for high-throughput quality control laboratories in the food manufacturing industry.

1.4 Advantages of HPLC

Liquid chromatography (LC) is an analytical separation method that separates components using a liquid mobile phase and a solid stationary phase. HPLC is an advanced method of LC that enables high resolving power under higher pressure compared to conventional LC. In normal-phase chromatography, a polar stationary phase and a less polar mobile phase are used. In reversed-phase chromatography, a nonpolar stationary phase and a polar mobile phase are used for the separation of organic compounds. Separated components are then detected using various techniques such as mass spectrometry (MS), FD, ultraviolet (UV) or ultraviolet-visible detectors (UV-Vis), and DAD [7].

When analyzing natural products, HPLC offers several advantages over other methods. It is highly sensitive, allowing detection of low concentrations. Its selectivity and versatility enable the separation of similar components, which is particularly beneficial for distinguishing caffeine and theobromine. For industrial purposes, quantification is essential, and HPLC facilitates accurate quantification of individual components in a mixture. Efficiency is another key factor when selecting an analytical method. HPLC is efficient and easily automated, allowing the analysis of multiple samples in a manner that reduces human errors. One of its most important features is compatibility with various detectors, as mentioned above, which enables fine-tuning and optimization of the method. More importantly, HPLC provides reliable data for quality control and quality assurance of industrially manufactured products.

1.5 Review Scope and Objectives

This review covers HPLC methods published between 2001 and 2023, encompassing both reversed-phase and normal-phase chromatography. Following a thorough literature survey, nine articles were selected for detailed comparison based on their relevance, analytical rigor, and applicability to industrial settings. This review aims to systematically evaluate and compare published HPLC methods for theobromine determination in cocoa products, with the specific objective of identifying an analytical approach that balances analytical performance (sensitivity, resolution, accuracy), operational simplicity (rapid sample preparation), and cost-effectiveness for routine implementation in industrial quality control laboratories.

2. MATERIALS AND METHODOLOGY

2.1 Search Criteria

A literature review was conducted using the following scientific databases: PubMed, Google Scholar, Web of Science, and ScienceDirect, covering publications from 2001 to 2023. Research articles were selected based on relevant terms appearing in the title, abstract, and author keywords, and only English-language publications were considered. The following search strings were used to retrieve relevant literature: ("Theobromine" AND "HPLC") OR "high-performance liquid chromatography", "Theobromine" AND ("cocoa" OR "chocolate" OR "cocoa products"), "Theobromine determination" AND "liquid chromatography", "Theobromine" AND "sample preparation" AND ("ultrasonic" OR "heat treatment"), "Methylxanthines" AND "cocoa" AND "chromatographic separation". This search yielded a total of 87 articles. After removing duplicates and applying the inclusion and exclusion criteria, 24 articles underwent full-text

assessment. From these, nine articles were selected for detailed comparison based on the justifications outlined in Section 2.3.

2.2 Inclusion and Exclusion Criteria

Articles were selected for this review based on predefined inclusion and exclusion criteria. The inclusion criteria required that articles be peer-reviewed, published between January 2001 and December 2023, and written in English. Studies had to focus on the determination of theobromine in cocoa-based products, including cocoa beans, cocoa powder, cocoa liquor, and chocolate products. Only articles employing HPLC as the primary analytical technique were considered, and studies had to report clear chromatographic conditions such as mobile phase composition, column type, and detection method. The exclusion criteria excluded studies analyzing theobromine in non-food matrices without direct application to cocoa products. Articles using only techniques other than HPLC were excluded. Only primary research articles were included, and conference abstracts, book chapters, and review articles were excluded. Finally, studies lacking sufficient methodological detail for meaningful comparison were also excluded.

2.3 Justification for Selection of Nine Articles

The articles selected for this review were required to meet the following objectives, as established in the introduction. Industrial applicability was considered with suitability for routine quality control in food manufacturing laboratories, balancing analytical performance with operational simplicity and cost-effectiveness. These included the resolution of structurally similar methylxanthines, practical sample preparation methods focusing on simple, rapid extraction techniques such as ultrasonic-assisted or heat-assisted extraction rather than time-consuming conventional procedures, and appropriate detection was focused on for reliable distinction of theobromine from interfering substances, with consideration of cost-effectiveness for industrial use. The nine articles were specifically justified based on the following criteria. They collectively represent a range of detection methods (UV-Vis, DAD, and MS), enabling meaningful comparison across detection techniques. They include diverse sample preparation approaches such as conventional solvent extraction, ultrasonic-assisted extraction, and heat treatment methods, allowing evaluation of industrial practicality. They cover a variety of cocoa matrices such as cocoa beans, cocoa powder, cocoa liquor, dark chocolate, milk chocolate, and white chocolate. Also, they originate from different research groups across Asia, Europe, and America, published between 2001 and 2023, ensuring the review captures methodological evolution. Each selected article provides sufficient analytical detail, such as mobile phase composition, flow rate, column specifications, detection parameters, and validation data, including linearity, LOD, LOQ, and recovery. Finally, articles were prioritized if they explicitly discussed or targeted routine industrial quality control applications rather than purely research-oriented methods.

3. RESULTS AND DISCUSSION

These studies were analyzed based on sample preparation techniques, chromatographic conditions (column type, mobile phase composition, flow rate), detection methods, recovery data, and industrial applicability. Table 01 provides a summarized overview of the analytical parameters employed in each study. A detailed comparative discussion of these parameters, including their strengths and limitations for routine quality control in the food manufacturing industry, is as follows.

3.1 Sample Preparation

Sample preparation methods varied considerably in complexity and time. Naik (2001) [1] and Brunetto et al. (2007) [7] used Soxhlet extraction with petroleum ether for 16 hours and 4 hours, respectively, which are time-consuming and less suitable for routine industrial use. Notably, Naik (2001) [1] employed a Sep-Pak C18 cartridge to purify the cocoa extract before injection onto the HPLC C18 column, a step that increases column life and reduces analysis time. The recovery range before and after passing through the Sep-

Table 1. Summary of HPLC analytical methods for the determination of theobromine in cocoa products

Article	Sample preparation	LC Column	Mobile phase	Flow rate/ ml/min	Detector	Injection volume	Column Temperature	Sample concentration	LOD	LOQ	Linearity
Naik (2001) [1]	Ground powders of fermented and dried cocoa beans were extracted in a Soxhlet using petroleum ether (40-60 °C) for 16 h	Sep-Pak C18 cartridge/ C18 reverse-phase column	Methanol and water (20:80)	1.0	UV at 274 nm	5-20 µl	-	Working standard = 0.08 µg/µL	-	-	-
Caudle et al. (2001) [12]	Cocoa-based Cereals were ground and extracted using Petroleum ether, mixing with the vortex and centrifuging	Reverse-phase HPLC C18 column (Phenomenex, Torrance, CA, USA)	Acetonitrile and water (10/90) acidified to pH 3 with phosphoric acid	1.0	UV at 280 nm	-	-	0.01 to 0.09 mg/ml	-	-	-

Brunetto et al. (2007) [7]	Ground powders of dried cocoa beans were extracted in a Soxhlet using petroleum ether (40-60 °C) for 4 h	Reversed-phase C18 Nova-Pak column (150 mm · 3.9 mm, 4 mm)	Methanol and water (20:80)	1.4	UV-DAD at 274 nm	20 µl	22 °C	2.0 to 20.0 µg/ml	0.10 µg/ml	0.50 µg/ml	r > 0.9901
Srdjenović et al. (2008) [8]	Chocolate products were extracted for 30 min at 60°C in an ultrasonic bath	Zorbax Eclipse XDB-C8 reversed-phase column (4.6 × 150 mm, 5-µm particle size)	water–THF (0.1% THF in water, pH 8)–acetonitrile (90:10)	0.8	UV-DAD at 273 nm	5 µl	25 °C	0.5 to 100.0 µg/ml	0.20 µg/ml	0.50 µg/ml	r > 0.9999

Cabugsa et al. (2016) [4]	The mixtures of cocoa products and HPLC-grade water were heated for 25 minutes at 100°C, cooled and filtered	Zorbax Eclipse XDB - C18 reversed phase Analytical 4.6 x 250 mm 5-micron.	A step gradient solvent system with acetonitrile and 1% phosphoric acid.	1.0	UV-DAD at 273 nm	-	35 °C	10 – 200 ppm	0.0727 ng/ml	1.0498 ng/ml	R^2 = 0.9999
Al-Qaim et al. (2018) [13]	Extracted using hot water (distilled water at 95 - 100 ºC for 5 min)	C18- reversed-phase column (length 250 mm, width 2.1 mm and diameter 5 µm).	Acetonitrile – MeOH (3:1, v/v) as mobile phase B and 0.1 % formic acid in deionized water as mobile phase A	0.3	Time of Flight - MS	30 µl	-	0.3 – 400 mg/L	-	0.15 µg/ml	R^2 = 0.999

Mladenovic et al. (2018) [14]	Cocoa-based Cereals were ground and extracted using Petroleum ether, mixing with the vortex and centrifuging	Waters Acquity UPLC BEH C18 reversed-phase column-1.7 µm pore size and 2.1 mm by 50 mm dimensions	A gradient program was set up with HPLC-grade water and acetonitrile both containing 0.1% formic acid.	-	MS	-	50 °C	~50 – 100 ng/L	-	LLOQ = 15 ng/ml, ULOQ = 1000 ng/ml	R^2 = 0.9979
Gray et al. (2019) [5]	Chocolate products were extracted for 20 minutes at room temperature in the ultrasonic bath	Luna C18 (2), 250mm × 4.60mm, 5 microns reversed-phase column (Phenomenex, Cheshire, UK)	90% 0.0125mol/l potassium dihydrogen phosphate, pH 3.5, 10% acetonitrile	1.0	UV at 278 nm	20 µl	30 °C	-	-	0.15 µg/ml	Linearity range of 27 to 307mg/kg
		Atlantis T3 C18 reversed phase, 3µm, 2.1mm x100mm column (Waters, Hertfordshire, UK)	Gradient elution of 12 mM ammonium formate in methanol/12 mM ammonium formate in water	0.2	MS	20 µl	25 °C	-	-	0.15 µg/ml	Linearity range of 27 to 307mg/kg

Gonzales-Yépez et al. (2023) [9]	Chocolate products were extracted for 30 minutes at 60 °C in the ultrasonic bath	Reversed phase purospher STAR RP-8 column (5µm particle size, i.d. 4:6× 150mm)	Isocratic elution with the mobile phase water-THF(A) (0.1% THF in water, pH 8)-ace tonitrile (B) (90:10, v/v). The pH was adjusted with 0.1M NaOH.	0.8	UV-DAD at 273 nm	-	25 °C	-	-	-	-

Pak cartridge was 96.92–99.90% and 96.11–100.19%, respectively. In contrast, Caudle et al. (2001) [12] and Mladenovic et al. (2018) [14] employed a simpler approach using petroleum ether with vortex mixing and centrifugation, reducing preparation time significantly. Srdjenović et al. (2008) [8], Gray et al. (2019) [5], and Gonzales-Yépez et al. (2023)[9] utilized ultrasonic bath extraction, offering faster alternatives. Cabugsa et al. (2016) [4] used heat extraction at 100°C for 25 minutes followed by filtration, while Al-Qaim et al. (2018) [13] employed the simplest and fastest method, hot water extraction at 95–100°C for only 5 minutes.

3.2 Chromatographic Columns

The majority of studies employed reversed-phase C18 columns. Naik (2001) [1] used a Sep-Pak C18 cartridge coupled with a C18 column. Caudle et al. (2001) [12] and Brunetto et al. (2007) [7] used Phenomenex C18 and Nova-Pak C18 columns, respectively. Cabugsa et al. (2016) [4] and Al-Qaim et al. (2018) [13] also utilized C18 columns from Zorbax and a generic C18, respectively. Srdjenović et al. (2008) [8] uniquely employed a Zorbax Eclipse XDB-C8 column (150 × 4.6 mm, 5 µm), which successfully resolved theobromine from caffeine. Mladenovic et al. (2018) [14] used a Waters Acquity UPLC BEH C18 column (50 × 2.1 mm, 1.7 µm), which, due to its smaller particle size, enabled faster separations. Gray et al. (2019) [5] compared two columns: a Phenomenex Luna C18(2) (250 × 4.6 mm, 5 µm) for UV detection and a Waters Atlantis T3 C18 (100 × 2.1 mm, 3 µm) for MS detection, and Gonzales-Yépez et al. (2023) [9] used a reversed-phase Purospher STAR RP-8 column (150 × 4.6 mm, 5 µm particle size).

3.3 Mobile Phase Composition

Mobile phases typically consisted of water mixed with methanol or acetonitrile, often acidified. Naik (2001) [1] and Brunetto et al. (2007) [7] used a simple isocratic mixture of methanol and water (20:80). Caudle et al (2001) [12] used acetonitrile and water (10:90) adjusted to pH 3 with phosphoric acid. Srdjenović et al. (2008) [8] employed a mixture of water with 0.1% tetrahydrofuran (THF) at pH 8 and acetonitrile (90:10). Cabugsa et al. (2016) [4] used a step gradient of acetonitrile with 1% phosphoric acid. Al-Qaim et al. (2018) [13] used a gradient with mobile phase A (0.1% formic acid in deionized water) and mobile phase B (acetonitrile: methanol at 3:1). Mladenovic et al. (2018) [14] employed a gradient of water and acetonitrile, both containing 0.1% formic acid. Gray et al. (2019) [5] used 90% potassium dihydrogen phosphate (0.0125 M, pH 3.5) with 10% acetonitrile for UV detection, and for MS detection, a gradient of 12 mM ammonium formate in methanol and water. Gonzales-Yépez et al. (2023) [9] also used an isocratic mobile phase consisting of water-THF (A) (0.1% THF in water, pH 8 adjusted with 0.1 M NaOH) and acetonitrile (B) in a ratio of 90:10 (v/v)

3.4 Flow Rate

Flow rates varied according to column dimensions and detector requirements. Naik (2001) [1] and Caudle et al. (2001) [12] used 1.0 mL/min. Brunetto et al. (2007) [7] used a higher flow rate of 1.4 mL/min. Srdjenović et al. (2008) [8] and Gonzales-Yépez et al. (2023) [9] used 0.8 mL/min. Cabugsa et al. (2016) [4] employed a gradient flow varying from 0.5 to 1.5 mL/min. Al-Qaim et al. (2018) [13] used a low flow rate of 0.3 mL/min, typical for MS compatibility. Mladenovic et al. (2018) [14] did not report the flow rate. Gray et al. (2019) [5] used 1.0 mL/min for the UV method and 0.2 mL/min for the MS method, the latter being the lowest among all studies.

3.5 Detection Methods and Recovery Data

Detection methods included MS, UV, DAD, and TOF-MS, with recovery rates varying across studies. Naik (2001) [1], Caudle et al. (2001) [12], and Mladenovic et al. (2018) [14] used standard MS detection. Naik (2001) [1] reported recoveries of 96.92–99.90% before and 96.11–100.19% after Sep-Pak purification. Caudle et al. (2001) [12] achieved an excellent recovery of 99.6%. However, Mladenovic et al. (2018) [14] reported a relatively low recovery of 85%, despite being accurate enough for cocoa processing applications.

Brunetto et al. (2007) [7], Srdjenović et al. (2008) [8], Gray et al. (2019) [5], Cabugsa et al. (2016) [4], and Gonzales-Yépez et al. (2023) [9] used UV detection. Brunetto et al. (2007) [7] reported a recovery of 95.8%, with the method allowing simultaneous sample processing and analysis at a frequency of 8 samples per hour. Srdjenović et al. (2008) [8] achieved outstanding recoveries ranging from 100.20% to 100.42%, and the authors noted that the method can be used for routine quality control of foods, drinks, and herbal products. Gray et al. (2019) [5] provided an accurate determination of theobromine in the range of 27 to 307 mg/kg.

Cabugsa et al. (2016) [4] used a DAD with a step gradient, reporting a recovery of 118.68% (±3.38%). Despite the high recovery value, the authors claimed high accuracy and repeatability with the use of a solvent gradient.

Al-Qaim et al. (2018) [13] employed time-of-flight (TOF)-MS, which provides high-resolution mass data, with recoveries ranging from 94% to 110%. The method was reported as accurate and sensitive enough to detect theobromine in beverages using a solvent gradient.

4. CONCLUSION

This review evaluated nine HPLC methods for the determination of theobromine in cocoa and cocoa-based products. Based on the comparative analysis of sample preparation, chromatographic conditions, detection methods, and recovery data, the following recommendations are made for developing a feasible analytical method suitable for routine quality control in the food manufacturing industry. For chromatographic separation, a reversed-phase C18 column with a guard column is recommended, as it prolongs column life and provides adequate resolution of theobromine from structurally similar caffeine. For the mobile phase, a mixture of water, methanol, and acetonitrile acidified with phosphoric or formic acid is commonly used. Gradient elution is recommended when baseline separation of theobromine from caffeine is required. For sample preparation, a simple ultrasonic bath extraction is ideal for industrial use, offering significantly faster processing than conventional Soxhlet extraction while maintaining excellent recoveries. Heat-assisted extraction provides an even faster alternative but requires careful optimization. For detection, UV detection is sufficient for routine quality control when combined with proper chromatographic separation. However, mass spectrometry is recommended for applications requiring higher sensitivity, faster analysis, or analysis of complex matrices where co-elution is a concern, despite the higher instrument cost. In summary, a feasible and efficient analytical method for theobromine determination in cocoa products can be developed by combining a reversed-phase C18 column with a guard column, an acidified water/methanol/acetonitrile mobile phase, ultrasonic or heat-assisted sample preparation, and either UV or MS detection, depending on industrial needs and budget.

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