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HPLC-DAD analysis of functional dietary supplements followed by liquid–liquid microextraction-assisted FTIR identification of IR-active ingredients

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Abstract

Accurate identification of active ingredients in dietary supplements and functional beverages is essential for ensuring product quality and consumer safety. Fourier transform infrared (FTIR) spectrometry, when coupled with high performance liquid chromatography (HPLC), provides molecular information for compound characterization but is limited by solvent interference. This study presents a liquid–liquid microextraction (LLME)–assisted HPLC–FTIR method for isolating and identifying bioactive

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compounds in complex supplement matrices. HPLC fractions of a branched-chain amino acid beverage were subjected to LLME using infrared-transparent, non-polar solvents to remove mobile phase residues and buffer salts prior to FTIR analysis. Extraction parameters, solvent composition, and buffer effects were optimized and compared with conventional solvent evaporation. The LLME approach effectively eliminated solvent and salt interference, yielding clear spectra that enabled identification of citric acid, L-ascorbic acid, valine, leucine, isoleucine, and caffeine. The method uses standard laboratory equipment and is compatible with common chromatographic systems, offering a practical solution for routine supplement quality control and regulatory testing. This LLME-assisted HPLC–FTIR strategy provides a reliable pathway for infrared-based identification of active ingredients in complex food and pharmaceutical products.

Keywords: dietary supplements; bioactive compounds; hyphenated techniques; interface

1. Introduction

The global demand for dietary supplements and functional beverages has seen rapid growth due to increasing awareness of preventive healthcare, wellness, and sports performance enhancement [1]. However, the lack of consistent regulation across regions raises serious concerns about the safety, efficacy, and authenticity of these products [2-5]. Quality assurance of such

products is vital not only for consumer protection but also for compliance with evolving international standards and regulatory frameworks [6,7]. Functional drinks, such as branched-chain amino acid (BCAA)-enriched beverages, are commonly marketed with claims to improve energy, support muscle recovery, and promote overall health [8,9]. However, adverse effects of consuming these drinks have also been reported [10,11]. These products typically contain bioactive compounds such as citric acid, ascorbic acid, valine, leucine, isoleucine, L-carnitine, and caffeine. However, their unregulated composition, variability among manufacturers, and potential presence of undeclared or adulterated substances pose significant analytical challenges [12]. Moreover, the presence of other components, such as artificial sweeteners, preservatives, or potential contaminants, requires comprehensive analysis to ensure the overall safety and effectiveness of these products [13].

To ensure the integrity of supplement products, verifying label claims, and complying with regulatory standards, reliable methods for identifying and quantifying active ingredients in complex matrices are essential. One powerful tool in this regard is Fourier transform infrared (FTIR) spectrometry, which offers non-destructive analysis, rapid operation, and rich molecular information, providing valuable insights for manufacturers, regulators, and consumers. However, FTIR suffers from severe spectral interference when coupled with high performance liquid chromatography (HPLC), primarily due to the strong absorption of infrared (IR) radiation by

the mobile phase solvents [14,15]. This has historically limited its utility in hyphenated applications, especially when analysing real-world samples with multicomponent matrices or buffer-containing eluents.

To address these challenges, two main strategies have been proposed: online flow cell coupling and off-line solvent elimination. While flow cells enable real-time detection, their short optical path length often leads to poor sensitivity and background correction issues [16,17]. In contrast, solvent elimination techniques enhance detection by removing volatile components before FTIR detection, thus improving signal clarity and lowering limits of detection (LOD) [18-20]. Nevertheless, traditional solvent removal by evaporation or heating often fails in the presence of buffer salts, which leave behind interfering residues that compromise spectral interpretation.

In this study, we propose an innovative approach to solvent elimination in HPLC-FTIR analysis using liquid-liquid microextraction (LLME). LLME enables the selective extraction and preconcentration of analytes into non-polar, IR-transparent solvents; bypassing many of the limitations of evaporation-based methods. The technique not only improves analytical sensitivity but also eliminates buffer salt interference, allowing for accurate FTIR analysis of previously inaccessible samples. This method was applied to a commercially available BCAA functional beverage, and key parameters influencing extraction efficiency and spectral quality were optimized. The approach shows promise as a robust, cost-effective, and scalable bioanalytical method for supplement quality control, directly supporting

public health and regulatory efforts.

2. Experimental

2.1. Reagents and Chemicals

All reagents were of analytical grade or of the highest available purity. Caffeine, glucose, tetramethylammonium iodide, 1-bromoheptadecafluorooctane, perfluoropropyl iodide, perfluorohexyl iodide, ammonium formate, and ammonium acetate were purchased from Sigma-Aldrich (MO, USA). Citric acid, L-ascorbic acid, L-tartaric acid, salicylic acid, malic acid, valine, leucine, isoleucine, methanol 99% for gradient elution, acetonitrile 99% for gradient elution. Formic acid 85%, acetic acid 100%, phosphoric acid 85%, and ammonium dihydrogen phosphate were purchased from Merck KGaA (Darmstadt, Germany). Potassium bromide of the highest purity was applied in the preparation of the FTIR pellet and was obtained from Lachema (Brno, Czech Republic). Ultrapure water was prepared by a Water Pro PS system (Labconco, Kansas City, KS, USA). The commercial nutritional supplement Pro!Brands BCAA drink (First Class Brands Sweden AB, Sweden), was purchased from a local market. Pro!Brands BCAA drink contains water, CO_2, L-leucine, L-isoleucine, L-valine, acidity regulators, flavorings, caffeine, sweeteners such as sucralose and acesulfame K, and added vitamins including vitamin C and several B-vitamins.In this study, non-polar solvents, such as 1-bromoheptadecafluorooctane, perfluoropropyl iodide, and perfluorohexyl iodide, were employed for LLME after reverse-phase (RP) HPLC separation of the analytes. They were chosen because they

do not absorb IR radiation within the desired spectral range of 600-4000 cm^{-1}, thus eliminating interference with the analytes' IR spectra.

2.2. Instrumentation

The pH of the solutions and buffers was determined using a combined pH electrode (glass, Ag/AgCl) connected to an inoLAB pH meter model 720 (WTW, Germany). Solid substances were weighed on a Sartorius 1712MP8 analytical balance (Quebec, Canada). Degassing of solvents was achieved using an ultrasonic cleaner, Ultrazvuk s.r.o. (Slovakia). Vortexing of the solutions was performed using a Vevor Lab Dancer IKA (Staufen, Germany). IR spectra were recorded using an FTIR spectrometer from BOMEM Hartmann & Braun (Quebec, Canada) equipped with an IR-Plan SPECTRA-TECH analytical micro-scope (Oak Ridge, USA) and transflective accessories. The FTIR spectrometer was cooled with liquid nitrogen obtained from Messer Gas Co. (Bratislava, Slovakia). Win-Bomem Easy 3.04 software from Galactic Industries Corp. (Quebec, Canada) was used for spectra processing. A deuterated triglycine sulphate detector at a wavenumber interval of 4000–750 cm^{-1} (mid-IR region) with a resolution of 4 cm^{-1} was employed. Each spectrum was recorded by averaging the signals of 64 scans in reflection mode. Before each measurement, the background was scanned to avoid measuring absorption bands that could distort the IR spectrum of the sample. The measured IR spectra were exported and edited using KnowItAll Information System 2017 by Bio-Rad Laboratories, Inc. (UK) and PAS Spectrum Viewer Protea software (UK).

HPLC separations were carried out on an Agilent 1100 instrument, consisting of an Agilent 1100 pump, an Agilent UV 1100 detector (CA, USA), and a Rheodyne manual dual sample injector (CA, USA). A monolithic octadecyl silica analytical column, Chromolith Performance RP-18e (100 mm × 4.6 mm I.D.), purchased from Merck KGaA (Darmstadt, Germany) was used as the analytical column. The flow rate of the mobile phase was set at 1.0 mL min^{-1} and the volume of the injected solutions was 100 µL. The detection of the analytes was carried out at 285 nm. Isocratic elution mode was used for the HPLC separation of the analytes. The software ChemStation version B.04.03 for HPLC systems from Agilent (Germany) was used to process the chromatographic data. Sampling for FTIR analysis was performed by manually collecting fractions from the HPLC and transferring them into 1.5 mL Eppendorf vials. The collection of fractions started immediately after the fraction zone started passing through the detector and continued until the chromatographic peak reached its maximum.

2.3. Preparation of solutions

Stock solutions of the analytes, caffeine, citric acid, L-ascorbic acid, L-tartaric acid and amygdalin at 1.0 mmol L^{-1} were prepared into ultrapure water at concentrations of 0.1 and 0.5 mmol L^{-1}. For the study of the influence of common solvents used as mobile phases in HPLC on the IR spectra of the analytes, mixtures of water and methanol and water and acetonitrile at 40:60 (v/v %) were prepared. Also, for this study, three different buffer solutions at a concentration of 10 mmol L^{-1} were prepared. These included formic

acid/ammonium formate at pH 3.6, acetic acid/ammonium acetate at pH 4.2, and phosphoric acid/ammonium dihydrogen phosphate at pH 2.7.

The only treatment applied to the Pro!Brands BCAA drink sample before analysis was ultrasonication to remove dissolved gases. Subsequently, a 100 µL aliquot of the drink was injected into the HPLC.

2.3.1. Liquid-liquid microextraction and traditional solvent elimination

For performing LLME, 1.0 mL of the collected HPLC fraction containing the analyte was placed in a 2 mL vial. Then, 20 µL of non-polar extraction solvent,1-bromoheptadecafluorooctane was rapidly injected into it using a microsyringe. The solution was then vortexed for 5 min at 3000 rpm. After equilibration, the non-polar extract phase was withdrawn by a microsyringe and transferred to an aluminium reflection substrate for further FTIR analysis. To compare with the LLME method, solvent elimination by evaporation with a stream of inert gas and heating was used. For this purpose, we fabricated a special interface consisting of two separable tubes. One end of the first tube was narrower, and the second tube had a hole that accommodated a cap with an aluminium reflective substrate (Figure 1). The solvent was subsequently removed by heating the interface to 100 °C under a stream of nitrogen gas for 1 to 10 min. These conditions were used as the final experimental conditions.

3. Results and Discussion

To evaluate the HPLC-FTIR method for real sample analysis using solvent elimination, three sets of experiments were performed. The first investigated the effect of substrate on the IR spectra of analytes to identify the optimal analytical conditions. The second examined the influence of mobile phases and buffer solutions. Finally, the third compared the effectiveness of LLME to solvent elimination by evaporation with different mobile phases, evaluating their impacts on the analytes IR spectra.

3.1. Study of Parameters Affecting IR Spectra of Analytes by Transflective Technique

3.1.1. Influence of the surface of transflective substrate material

The selection of an appropriate material for transflective substrate surface is crucial for obtaining an accurate IR spectrum of the analytes. Key factors influencing this choice include the physical properties, structure, surface smoothness and cleanliness of the substrate. Additionally, it is important to consider factors such as sample preparation, including solvent type, pH value of the sample, buffer type, temperature, and HPLC separation conditions.

Germanium crystal (Ge crystal), zinc selenide crystal (ZnSe crystal), aluminium foil (Al foil), aluminium sheet (Al sheet), silver sheet (Ag sheet), stainless steel sheet (SS sheet), and polished golden layer (Au layer) were chosen as transflective substrates in this study. The thickness of the transflective substrates used was approximately 4 mm for the crystal substrates and about 0.3 mm for the sheet, layer, and plate substrates. The

IR spectra of caffeine were measured on the studied substrates and compared with each other. The lowest background IR spectra in the wavenumber ranges of 800–2000 cm^{-1} and 2300–2400 cm^{-1} were observed for the Ge crystal, ZnSe crystal, Al foil, and Al sheet. The absorption bands at 4000 to 3500 cm^{-1} and 2000 to 1400 cm^{-1} are attributed to water vapour and carbon dioxide. The occurrence of low intensity absorption bands can be attributed to other contaminants present in the surrounding environment or on the surface of the transflective substrate. Small differences in the background IR spectra of the individual transflective substrates are noted at 2900 cm^{-1}. The most intense absorption band at this wavelength appears on the IR background spectrum of the ZnSe crystal. This phenomenon can be explained by the fact that the ZnSe crystal can absorb or reflect IR radiation more strongly in this IR region. This leads to an enhancement of the corresponding background band. This behaviour may be due to surface properties, contaminant residues, or the internal structure of the material. Therefore, this interference needs to be considered when selecting a suitable transflective substrate for FTIR analysis. Similar differences were also found at 1300 cm^{-1}, where the absorption band in the IR spectrum of the Au background layer is the most intense. In each IR spectrum of the transflective substrate, there is a double absorption band between 2300 and 2400 cm^{-1}, which is due to a mechanical error of the FTIR spectrometer. To determine the measured values of the radiation intensity incident on the detector, the percent of incident radiation to the detector was measured for all substrates.

These measured values are depicted in Figure 2. As can be seen, more IR radiation can be reflected by glossy and polished materials, among them, Al foil provides the highest reflection.

In order to identify the best substrate for the analysis of analytes in the real sample, the IR spectra of the solid caffeine on these seven substrates were measured (Figure 3). To confirm this selection, the IR spectra of ascorbic acid as another analyte were recorded on the same substrate which confirmed our selection of substrate. Based on these facts, Al foil was selected as the suitable substrate for further FTIR analyses.

3.1.2. Influence different mobile phase

This section examines the effect of commonly used mobile phase composition on hyphenated reverse-phase HPLC-FTIR analyses. When using this technique, selecting non-polar solvents with low boiling points is crucial. Therefore, polar solvents must be removed to avoid their absorption bands interfering with the IR spectrum. Notably, impurities are more common in polar solvents. Methanol, for example, exhibits small absorption bands at 2950, 1950, 1300, 1000 and 750 cm^{-1}, which are due to the presence of impurities in it. Additionally, solvents may contain other interferences such as dust, moisture from the air, and mixtures of organic compounds. These can cause interference in the IR spectrum of the analyte.

To investigate the effect of various solvents used as mobile phases for separation on the IR spectra of analytes, the first two of the most common mobile phases were selected. Methanol–water and acetonitrile–water

mixtures were then prepared in a 60:40 volume ratio. To each solution, 1.5 mL of ascorbic acid was added to prepare aqueous solutions at concentrations of 0.1 and 1.0 mmol L^{-1}. Subsequently, 50 µL aliquots were taken and the solvents were evaporated by the solvent elimination technique using an inert stream of nitrogen gas and heating. Solid ascorbic acid crystals were identified by FTIR spectrometry with a micro-scope using the reflectance-absorption (R-A) technique on an Al foil substrate. The advantage of using microscope is that it improves the quality of the IR spectrum of the analyte by allowing easier focusing. All IR spectra of ascorbic acid analytes are well distinguished, and impurities found in the mobile phase had no impact on the spectra quality. The only difference in the spectra of 0.1 and 1 mmol L^{-1} solutions is in the intensity of certain characteristic absorption bands of ascorbic acid. There were minimal differences between the IR spectra of the samples prepared using two mobile phases.

3.1.3. Influence of presence of buffers in the mobile phase

The presence of buffer in the mobile phase poses a problem when HPLC is hyphenated with FTIR. This is because salt crystals mostly form when the solvent is eliminated, whereas weak acids or weak bases evaporate due to their low boiling points. The characteristic absorption bands of these salt crystals can interfere with the IR spectrum of an analyte. The aim of the next experiment was to investigate the effect of different buffer solutions on the IR spectra of the ascorbic acid after solvent elimination. For this purpose, three buffer solutions—ammonia and formic acid (formate), ammonia and

acetic acid (acetate), and ammonia and phosphoric acid (phosphate)—were prepared. These buffers are the same as those mentioned in the “Preparation of solutions” section. The worst quality of IR spectrum was observed in the solution containing phosphate buffer, because the IR spectrum of this solution completely overlaps with the FTIR spectrum of the ascorbic acid. In contrast, IR spectra of ascorbic acid obtained in the solvent mixture of methanol and formate or acetate buffer solutions are characterised by intense characteristic absorption bands of ascorbic acid. However, even in these cases the following spectral interferences may occur. The formate and acetate buffer solutions have a characteristic C-H bond vibrational absorption band at 2900 to 3000 cm^{-1} which may overlap the characteristic C-H bond vibrational absorption band for ascorbic acid. Further, the valence vibration of the ascorbic acid bond from 3000 to 3600 cm^{-1} may overlap an absorption band of the valence vibration of the N-H bond of formate and acetate buffer solutions. Interference of the absorption bands associated with vibrations of the C=O and C-N bonds at 1600 to 1700 cm^{-1} from formate and acetate buffer solutions with the absorption band of the C=O bond of ascorbic acid was also observed.

3.1.4. Solvent elimination by liquid-liquid microextraction

Due to the above-mentioned limitations of using more complex solvents, which also contained buffer solutions, we sought to apply LLME as an alternative. It also has the capability of removing solvents that could not be removed by the other solvent elimination methods, as well as salts of the

buffer solutions. Such solvents include dimethyl sulfoxide (DMSO), tetrahydrofuran (THF), N,N-dimethylformamide (DMF), acetamide, ethylene glycol, propylene glycol, and selected buffers. These solvents are characterised by their high boiling point, low volatility or strong hygroscopicity, which makes their complete removal by traditional methods impossible.

To perform LLME successfully and extract analytes with the highest efficiency, parameters affecting microextraction should be optimized, with the selection of the extracting solvent being the most important. Since samples were prepared in a solvent mixture the extraction solvent needs to be a suitable non-polar reagent, which also has low absorption of IR radiation. Therefore, 1-bromoheptadecafluorooctane, perfluoropropyl iodide and perfluorohexyl iodide were selected as extracting solvents. Nine series of standard solutions of caffeine, glucose, tetramethylammonium iodide, salicylic acid, vitamin B12, malic acid, bis(cyclohexanone)oxaladihydrazone, amygdalin and ascorbic acid were prepared in water within the concentration range of 1 to 30 mg L^{-1}. By using an ultraviolet spectrophotometer, a calibration curve was plotted to determine extraction recoveries of non-polar extraction reagents.

LLME was performed on the collected fractions as described in “Liquid-liquid microextraction and traditional solvent elimination” section. Twenty µL of the non-polar extract phase was removed and transferred to a microcell of the spectrophotometer for recovery determination. Extraction recoveries were

calculated based on the amount of analyte extracted into the non-polar extract phase from the solvent mixture. Table 1 summarizes the extraction recoveries obtained using the three non-polar solvents evaluated for LLME. These results highlight the differential extraction capabilities of each solvent and justify the selection of the most efficient solvent for subsequent optimization steps. The highest extraction recovery was achieved by 1-bromoheptadecafluorooctane for all analytes studied. Despite the lower values, the extraction recovery of the analytes for the purpose of their identification was sufficient because we obtained pure analytes without interferents. To compare the efficiency of LLME and solvent elimination by evaporation for separating the solvent from the sample, ascorbic acid was chosen as the analyte. It had one of the highest extraction recovery rates when a non-polar extraction reagent was selected. Buffer was added to the solvent mixture for the purpose of evaluating its effect on the solvent elimination by LLME or both LLME and the traditional solvent elimination. Two mobile phases with different compositions were used in this study. The first (mobile phase 1) contained water and ammonium acetate buffer (pH 4.2) in a ratio of 60:40 (v/v %). The second (mobile phase 2) was composed of water, methanol, acetonitrile, and ammonium acetate buffer (pH 4.2) in a ratio of 30:20:20:30 (v/v %). With this study, we have confirmed the usefulness of LLME as an alternative or complementary method for removing solvents from samples. Figure 4 shows the IR spectra of ascorbic acid obtained after the solvent elimination either with LLME alone or by

combining LLME with solvent elimination by evaporation.

For simple solvents such as water and methanol, solvent elimination by evaporation was sufficient for their complete elimination. We had to use LLME for the samples that contained buffer solutions. We were not able to remove the salts of the buffer solution using solvent elimination by evaporation.For solvents containing more than five components, we were forced to use both methods. First, several solvent components were removed by evaporation, followed by LLME to extract the sample into the non-polar extraction phase.There were several reasons why we used both methods for multicomponent solvents. The first reason was the limited extraction of the sample into a non-polar extraction agent. The second reason was that even if the extraction was successful, establishing chemical equilibrium was time-consuming. Table 2 shows a comparison of LLME and solvent elimination by evaporation, singly or in combination, on the elimination efficiency of different solvents prior to FTIR analysis of ascorbic acid. This comparison demonstrates how sample preparation strategies influence solvent elimination efficiency and supports the choice of the method that provides the cleanest IR spectra.

4. Real sample analysis

Off-line coupling of HPLC and FTIR was used for the real sample analysis, using both LLME and solvent elimination by evaporation. Nutritional supplement, Pro!Brands BCAA drink was selected as the real sample. For this real sample, three different mobile phases were used to show the differences

in using different solvents with different methods to separate the solvent from the sample. The first mobile phase contained water and methanol in a ratio of 70:30 (v/v %). The second was composed of water, methanol, and acetonitrile in a ratio of 60:30:10 (v/v %). The third consisted of water, methanol, acetonitrile, and ammonium acetate buffer (pH 4.2) in a ratio of 50:20:10:20 (v/v %). Citric acid, L-ascorbic acid, valine, leucine, isoleucine, and caffeine were separated from the sample. The second mobile phase was found to be most effective for the separation of citric acid and L-ascorbic acid. The third mobile phase was most effective for the separation of valine, leucine, isoleucine, and caffeine. Reference materials were used to determine the order of separation of individual components of the sample under the same conditions.

For each separation, 1.0 mL of the selected isoleucine fraction was collected, and solvent elimination was performed by three solvent elimination methods: 1. solvent elimination by evaporation, 2. LLME, and 3. a combination of both methods. The first two solvent elimination methods (evaporation and LLME) were performed as described in the previous sections. In the case of combining these methods, each collected 1.0 mL fraction was first exposed to a stream of inert nitrogen and heated to 100 °C. This was done to evaporate approximately half of the solution and reduce the content of low-boiling components from the solvent, preventing degradation and crystallization of the sample or solvent. The residual liquid phase was then microextracted with 20 µL of 1-bromoheptadecafluorooctane. The non-polar extract phase with

the sample was transferred onto an aluminium foil using a syringe and analysed by FTIR. These procedures were repeated under the same conditions for all three mobile phases (Figure 5).

As with the first mobile phase, for all the three methods of solvent elimination, a thick film of isoleucine was obtained on the aluminium foil. Due to the simple composition of this mobile phase, solvent elimination by evaporation seems sufficient. However, the second and third solvents contained a buffer which left behind salt crystals after the traditional solvent elimination. By using LLME, this problem was eliminated. Using a combination of solvent elimination by evaporation and LLME, the same result as using only LLME was obtained. Nevertheless, the peaks were larger in the IR spectrum due to preconcentration that occurred from reducing the sample volume.Photos of prepared isoleucine are provided as evidence that the solvent elimination by the combination of solvent elimination by evaporation and LLME was successful to obtain a pure solid analyte (Figure S1 in Suppl. Materials). On the left (A) is a standard of isoleucine with a high concentration, which formed a crystallized film. On the right (B) is isoleucine from a sample that formed a small thin film after the solvent elimination. The isoleucine shown in the photos was obtained from the third mobile phase. This preconcentration clearly enhanced peaks at 3200 to 3600 cm^{-1} (Figure 5), where there is a wide absorption band of the valence vibration of the O-H bond. At 1200 to 1700 cm^{-1}, characteristic absorption bands of "fingerprints" are not as large as in the other IR spectra of isoleucine. For each component

separated from the sample, the entire process of real sample analysis has been conducted.

5. Discussion

Developing a reliable interface between chromatographic separation and FTIR detection has been a persistent challenge in analytical chemistry, especially for complex matrices such as functional beverages. In this work, LLME served as a critical sample preparation step, enhancing the compatibility of FTIR detection with analytes eluted from an HPLC column. This pioneer study enabled the successful identification of six key compounds—valine, leucine, isoleucine, citric acid, ascorbic acid, and caffeine—demonstrating its potential as a practical analytical platform. The effectiveness of LLME in removing interfering buffers and aqueous solvents addresses a significant limitation in conventional HPLC-FTIR setups. Previous studies have noted the difficulty of coupling water-based HPLC systems with FTIR due to strong IR absorption from water and salts [14,15]. In our method, analytes were efficiently transferred into an IR-transparent organic phase, allowing the collection of clean and interpretable spectra. As shown in Figure 4, the IR spectrum clearly shows the characteristic vibrational bands of the analyte, ascorbic, with almost no interference from the mobile phase. The method is succeeded in spectral identification even for compounds with structurally similar backbones, such as BCAAs. This is particularly important given the widespread use of BCAAs in dietary supplements and the necessity of distinguishing them accurately for

regulatory compliance and consumer protection [2,6].

Another major advantage of this LLME-HPLC-FTIR approach lies in its simplicity and accessibility. Unlike online HPLC-FTIR configurations that require specialized flow cells or salt-free mobile phases [16], our approach uses standard laboratory tools and commercially available solvents. This reduces both cost and technical barriers, making it suitable for routine quality control of supplements and functional foods, which are under increasing scrutiny for content verification.

Compared to well-established techniques such as high performance liquid chromatography-mass spectrometry (HPLC-MS), which offer high sensitivity and molecular specificity, the proposed LLME-assisted HPLC-FTIR method presents a practical alternative for routine qualitative analysis. HPLC-MS systems, while highly powerful, are associated with substantial operational costs, require highly trained personnel, and often demand elaborate sample preparation to reduce matrix effects. In contrast, the LLME-HPLC-FTIR approach enables rapid identification of analytes using more accessible instrumentation and avoids issues related to ion suppression or mass calibration. Although it does not match LODs of HPLC-MS, the method provides sufficient selectivity and spectral clarity for identifying major constituents in complex matrices. Its ability to eliminate solvent and buffer interference prior to FTIR detection makes it particularly useful in cases where rapid screening and structural fingerprinting are required.

One of the main limitations of the developed method is its lower efficiency and

selectivity. These are influenced by the narrow choice of a suitable organic extraction reagent that is immiscible with water and exhibits low absorption in the IR spectrum. Another limitation of the method is the use of offline fraction collection followed by manual LLME processing, which reduces automation potential and makes the method less suitable for routine analyses. The applicability of the developed method is also limited to the analytes that contain chromophoric groups enabling their UV-Vis detection during HPLC separation and IR-active functional groups suitable for recording IR spectra. However, in this study, our primary aim was to demonstrate the feasibility and analytical usefulness of coupling HPLC separation with LLME cleanup and FTIR fingerprinting. While the method proved effective for the analysed BCAA drink, it holds promise for broader application. LLME can be adapted to isolate a wide range of polar and non-polar compounds, potentially enabling FTIR-based screening of herbal products, pharmaceuticals, and even biofluids. Integration with techniques such as ATR-FTIR or hyphenated mass spectrometry could further enhance compound characterization, as previously suggested in pharmaceutical analysis workflows [19].

6. Conclusions

This study introduces a novel liquid-liquid microextraction technique integrated with HPLC-FTIR for the effective identification of active ingredients in functional supplements. The LLME approach successfully addresses the limitations of conventional solvent elimination methods by efficiently removing interfering solvents and salts, thereby enhancing

spectral clarity and analytical sensitivity. Applied to a branched-chain amino acids beverage, the method enabled the precise identification of compounds such as citric acid, L-ascorbic acid, valine, leucine, isoleucine, and caffeine. The technique's compatibility with IR-transparent non-polar solvents and minimal equipment requirements supports its implementation in routine pharmaceutical and biomedical laboratories. This advancement offers a robust, scalable, and cost-effective analytical tool for quality control and regulatory analysis of dietary supplements, functional beverages, and other biomedical products.

It is important to emphasize that the developed HPLC–LLME–FTIR method is not intended to replace HPLC–MS, which remains superior in terms of sensitivity, selectivity, structural elucidation, and quantitative performance. Instead, the proposed method should be viewed as a complementary, practical alternative for situations where rapid identification is required, non-volatile or non-MS-compatible buffers are necessary during chromatographic separation, or when a mass spectrometer is not available. The LLME clean-up and preconcentration step effectively removes mobile-phase solvents and inorganic salts, enabling clear IR fingerprint spectra at very low operational cost. Thus, while HPLC–MS remains the standard instrument for trace-level and comprehensive analyses, the HPLC–LLME–FTIR approach offers a simple, robust, and economical option for targeted quality-control and screening applications. Future efforts may focus on extending this approach to a broader range of clinically relevant compounds and therapeutic matrices.

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Authors' contributions

DP: conceptualization, investigation, writing – original draft, writing – review and editing. MK: literature search, writing – original draft, writing – review and editing. JJ: literature search, writing – original draft, writing – review and editing. RH: supervision, conceptualization, writing – original draft, writing – review and editing. All authors read and approved the final manuscript.

Data Availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

Competing interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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Captions to Figures and Tables

Figure 1. Fabricated interface that was used for solvent elimination in this research. (A) Whole apparatus with interface. (B) Detail of the interface. (C) Interface with scale.

Figure 2. Intensity of radiation falling into the detector by different R-A substrates.

Figure 3. IR spectra of caffeine on four different transflective substrates.

Figure 4. IR spectra of ascorbic acid (A) Standard. (B) After solvent elimination by evaporation and LLME from mobile phase 2. (C) Solvent elimination by evaporation and LLME from mobile phase 1. (D) LLME from mobile phase 2.

Figure 5. IR spectra of isoleucine obtained from the mobile phase after: (A) LLME. (B) solvent elimination by evaporation and LLME. (C) Solvent elimination by evaporation. The mobile phase contained water, methanol, acetonitrile, and ammonium acetate buffer (pH = 4.2) in a ratio of 50:20:10:20 (v/v %).

Table 1. Extraction recoveries (%) of analytes using three non-polar solvents employed for LLME.

Table 2. Comparison of LLME and solvent elimination by evaporation, singly or in combination, on the elimination efficiency of different solvents prior to FTIR analysis of ascorbic acid.

Table 1. Extraction recoveries (%) of analytes using three non-polar solvents employed for LLME.

Analyte	1-Bromoheptadeca-fluorooctane		Perfluoropropyl iodide		Perfluorohexyl iodide	
	E (%)	RSD (%)	E (%)	RSD (%)	E (%)	RSD (%)
Caffeine	40	2,5	21	3,5	15	15,7
Glucose	40	3,1	19	6,9	24	7,6
Tetramethylammonium iodide	56	3,1	31	12,1	27	11,5
Salicylic acid	33	3,2	22	7,4	16	4,6
Vitamin B12	40	4,1	29	3,6	31	10,7
Malic acid	37	4,7	26	8,8	25	7,5
Bis(cyclohexanone)oxaladihydrazone	34	3,7	22	4,8	12	5,2
Amygdalin	34	5,4	13	7,3	17	4,7

Ascorbic acid	52	3,1	28	4,2	1 5	5,8

E – extraction recovery; RSD - relative standard deviation for the number of measurements n = 3.

Table 2. Comparison of LLME and solvent elimination by evaporation, singly or in combination, on the elimination efficiency of different solvents prior to FTIR analysis of ascorbic acid.

Solvent elimination		Mobile phase	Extraction recovery (%)	Was the solvent elimination successful?
Evaporation	LLME*			
No	Yes	1	31	Yes
Yes	Yes	1	34	Yes
No	Yes	2	1	No
Yes	Yes	2	48	Yes

*with non-polar reagent 1-bromoheptadeca-fluorooctane; mobile phase 1: water and ammonium acetate buffer (pH = 4.2) in a ratio of 60:40 (v/v %) and mobile phase 2: water, methanol, acetonitrile, and ammonium acetate buffer (pH = 4.2) in a ratio of 30:20:20:30 (v/v %)