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HPLC CHARACTERIZATION OF WATER-SOLUBLE VITAMINS AND FREE AMINO ACIDS IN A DRY AQUEOUS EXTRACT OF POMEGRANATE (PUNICA GRANATUM L.) PEEL

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ABSTRACT

Pomegranate peel is an abundant plant-derived by-product that contains chemically diverse constituents and is increasingly investigated as a raw material for standardized herbal preparations. This study characterized selected water-soluble vitamins and free amino acids in a dry aqueous extract of pomegranate (Punica granatum L.) peel using high-performance liquid chromatography with diode-array detection. Vitamins were extracted from the dry material with 40% ethanol and separated on an Eclipse XDB C18 column. Free amino acids were isolated after precipitation of proteins and peptides with trichloroacetic acid, derivatized before analysis with phenylisothiocyanate, and separated on a Discovery HS C18 column. Six water-soluble vitamins were quantified. Folic acid (vitamin B9) was the most abundant at 98.84 µg/g, followed by ascorbic acid (38.55 µg/g), pyridoxine (34.90 µg/g), thiamine (28.90 µg/g), niacin (18.87 µg/g), and riboflavin (15.90 µg/g). Twenty free amino acids were reported, with a combined concentration of 14.456 mg/g. Aspartic acid (3.184 mg/g), histidine (1.735 mg/g), tyrosine (1.495 mg/g), leucine (1.390 mg/g), alanine (1.346 mg/g), and methionine (1.253 mg/g) were predominant. The chromatographic profiles support the use of vitamin and amino-acid composition as supplementary quality attributes for the extract. However, full pharmaceutical standardization will additionally require botanical authentication, batch-to-batch assessment, validated calibration and recovery data, and stability and contaminant testing.

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1. INTRODUCTION

Medicinal plant materials and their extracts are chemically complex products whose quality cannot be established by a single visual or chemical test. Their composition may vary with species identity, cultivar, geographical origin, harvest conditions, post-harvest handling, extraction solvent, extraction time, temperature, and storage. For this reason, internationally recognized guidance treats identity, purity, content, and analytical consistency as complementary elements of herbal-material quality control [1,2]. The European Pharmacopoeia likewise emphasizes clearly defined raw materials, validated analytical procedures, and scientifically justified specifications when herbal drugs and herbal preparations are described in monographs [3].

Pomegranate (Punica granatum L.) is a fruit-bearing species with a long history of dietary and traditional medicinal use. The peel constitutes a substantial fraction of the fruit and is commonly discarded during juice and food processing. At the same time, it is a concentrated source of hydrolysable tannins, phenolic acids, flavonoids,

and other polar constituents. Reviews of pomegranate chemistry and biological activity consistently describe the peel as one of the chemically richest anatomical fractions of the fruit [5-7]. This combination of abundance and chemical complexity makes pomegranate peel attractive for the development of extracts intended for pharmaceutical, nutraceutical, cosmetic, and food applications.

Much of the published research on pomegranate peel has focused on polyphenols, especially punicalagins, ellagic acid derivatives, gallic acid derivatives, and related tannins. Experimental studies have also demonstrated that peel extracts can show strong antioxidant activity in chemical and lipid-oxidation models [8,9]. Detailed HPLC-DAD-MS profiling has confirmed that pomegranate peel contains numerous phenolic compounds and that the phenolic pattern varies among peel, mesocarp, aril, and juice fractions [10]. These findings provide an important chemical foundation, but they do not describe the entire extract. Polar micronutrients and nitrogen-containing constituents may also contribute to its analytical identity and biological value.

Water-soluble vitamins are chemically heterogeneous molecules involved in redox reactions, one-carbon metabolism, energy production, amino-acid metabolism, connective-tissue synthesis, and other physiological processes. Their simultaneous determination is analytically challenging because they differ in polarity, ultraviolet absorbance, stability, and expected concentration. Reversed-phase HPLC with diode-array detection is widely used for multivitamin analysis when sample preparation, wavelength selection, chromatographic separation, and calibration are appropriately optimized [12,13]. In botanical matrices, these requirements are especially important because co-extracted compounds may interfere with peak identification or quantification.

Free amino acids are another potentially informative group of constituents. In addition to serving as protein building blocks, they participate in plant nitrogen transport, osmotic regulation, stress responses, and biosynthetic pathways. Their concentrations can therefore contribute to the chemical fingerprint of a plant-derived extract. Because native amino acids generally show weak ultraviolet absorbance, pre-column derivatization is commonly used. Phenylisothiocyanate reacts with amino groups to form stable phenylthiocarbamyl derivatives that can be separated by reversed-phase HPLC and detected in the ultraviolet range [14,15].

For an herbal extract, vitamin and amino-acid profiles should not be interpreted as complete proof of therapeutic efficacy. They are more appropriately regarded as compositional attributes that can support identification, comparability, and specification development. Regulatory-quality analytical work also requires evidence of selectivity, accuracy, precision, linearity, range, detection and quantification limits where relevant, and robustness [4]. The present study therefore had two linked aims: first, to report the qualitative and quantitative profiles of selected water-soluble vitamins and free amino acids in a dry aqueous pomegranate-peel extract; and second, to evaluate how these results may contribute to a broader standardization strategy while clearly identifying the additional evidence required for pharmaceutical quality control.

2. Materials and Methods

2.1 Study material and extract

The study material was a dry extract prepared from pomegranate (Punica granatum L.) peel. According to the available experimental description, the peel was dried, finely milled, extracted with water, and dried to obtain a powdered extract. The analytical work was performed at Tashkent Pharmaceutical Institute, Tashkent, Uzbekistan. The source record did not provide the collection locality, harvest date, cultivar, voucher specimen number, extraction ratio, extraction temperature, extraction duration, drying conditions, extraction yield, batch number, or storage history. These details should be added from the original laboratory documentation before the manuscript is submitted to a journal because they are necessary for reproducibility and botanical traceability [1-3].

2.2 Determination of water-soluble vitamins

The target analytes were thiamine (vitamin B1), riboflavin (vitamin B2), niacin (vitamin B3), pyridoxine (vitamin B6), folic acid (vitamin B9), and ascorbic acid (vitamin C). A 5-10 g portion of the dry extract was accurately weighed into a 300 mL flat-bottom flask, and 50 mL of 40% ethanol was added. The mixture was heated under reflux with continuous magnetic stirring for 1 h and then stirred at room temperature for an additional 2 h. After filtration, the residue was extracted twice more with 25 mL portions of 40% ethanol. The filtrates were combined in a 100 mL volumetric flask and brought to volume with 40% ethanol. The solution was centrifuged at 7000 rpm for 10 min, and the supernatant was used for HPLC analysis.

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Working standard solutions were prepared at a nominal concentration of 1 mg/mL. Chromatographic separation was performed with an Agilent Technologies 1200 HPLC system equipped with a diode-array detector. An Eclipse XDB C18 column (4.6 × 250 mm, 5 µm) was maintained at 25 °C. The mobile phase consisted of acetate buffer and acetonitrile under gradient-elution conditions. The flow rate was 0.8 mL/min, the injection volume was 5 µL, and detection was performed at 250 nm. These conditions are consistent with the general use of HPLC-DAD for multivitamin analysis, although full method validation information was not available in the source record [12,13].

2.3 Determination of free amino acids

Proteins and peptides in the aqueous sample solution were precipitated by mixing 1 mL of sample with 1 mL of 20% trichloroacetic acid. After 10 min, the mixture was centrifuged at 8000 rpm for 15 min. A 0.1 mL aliquot of the supernatant was freeze-dried. The residue was dissolved in a triethylamine-acetonitrile-water mixture (1:7:1, v/v/v) and evaporated to dryness. This neutralization step was repeated twice to remove residual acid.

The free amino acids were derivatized with phenylisothiocyanate to form phenylthiocarbamyl derivatives before chromatographic analysis, following the established analytical principle described for pre-column PITC derivatization [14,15]. The HPLC system was an Agilent Technologies 1200 instrument with diode-array detection. Separation was performed on a Discovery HS C18 column (75 × 4.6 mm). Mobile phase A was 0.14 M sodium acetate containing 0.05% triethylamine and adjusted to pH 6.4; mobile phase B was acetonitrile. A 55 min gradient program was used at a flow rate of 1.2 mL/min, and detection was carried out at 269 nm.

2.4 Replication, quantification, and data treatment

The source manuscript states that all analyses were performed in triplicate and that the results were reported as mean values. Individual replicate measurements, standard deviations, calibration equations, correlation coefficients, recovery data, peak-purity assessments, detection limits, quantification limits, and system-suitability results were not included. Accordingly, the present paper reports the available mean concentrations without inferential statistical comparisons. The amino-acid total was checked arithmetically and equals 14.456 mg/g. Relative contributions of individual amino acids were calculated as the concentration of each analyte divided by the summed concentration of all 20 reported amino acids.

Table 1. HPLC operating conditions used for vitamin and free-amino-acid analyses

Parameter	Water-soluble vitamins	Free amino acids
Instrument	Agilent Technologies 1200 HPLC-DAD	Agilent Technologies 1200 HPLC-DAD
Column	Eclipse XDB C18, 4.6 × 250 mm, 5 µm	Discovery HS C18, 75 × 4.6 mm
Mobile phase	Acetate buffer-acetonitrile	A: 0.14 M sodium acetate + 0.05% triethylamine, pH 6.4; B: acetonitrile
Elution	Gradient	Gradient, 55 min
Flow rate	0.8 mL/min	1.2 mL/min
Detection	250 nm	269 nm
Injection volume	5 µL	Not reported
Column temperature	25 °C	Not reported

3. Results

3.1 Water-soluble vitamin profile

Six water-soluble vitamins were quantified in the dry pomegranate-peel extract (Table 2). Folic acid was the dominant measured vitamin at 98.84 µg/g. Its concentration was approximately 2.6 times that of ascorbic acid and 2.8 times that of pyridoxine. Ascorbic acid (38.55 µg/g) and pyridoxine (34.90 µg/g) formed the second concentration tier, followed by thiamine (28.90 µg/g), niacin (18.87 µg/g), and riboflavin (15.90 µg/g). The standard-solution chromatogram is presented in Figure 1, and the chromatogram obtained for the extract is shown in Figure 2.

The chromatograms support the presence of multiple ultraviolet-absorbing constituents within the expected retention-time region. However, the extract chromatogram also contains a dense cluster of peaks and an elevated baseline in part of the run, indicating a chemically complex matrix. For publication-quality quantification, analyte identity should therefore be supported not only by retention-time matching but also

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by spectral peak-purity evaluation, standard addition or spiking, and validated recovery experiments. Vitamin B12 is labelled in the standard chromatogram, but no validated concentration for vitamin B12 was provided in the results table or source text; consequently, it is not reported as a quantified constituent of the extract.

Table 2. Water-soluble vitamins quantified in the dry pomegranate-peel extract

Analyte	Mean concentration (µg/g)
Thiamine (vitamin B1)	28.90
Riboflavin (vitamin B2)	15.90
Niacin (vitamin B3)	18.87
Pyridoxine (vitamin B6)	34.90
Folic acid (vitamin B9)	98.84
Ascorbic acid (vitamin C)	38.55

Note. *Values are means of triplicate analyses as reported in the source document; replicate-level values and dispersion statistics were not available.*

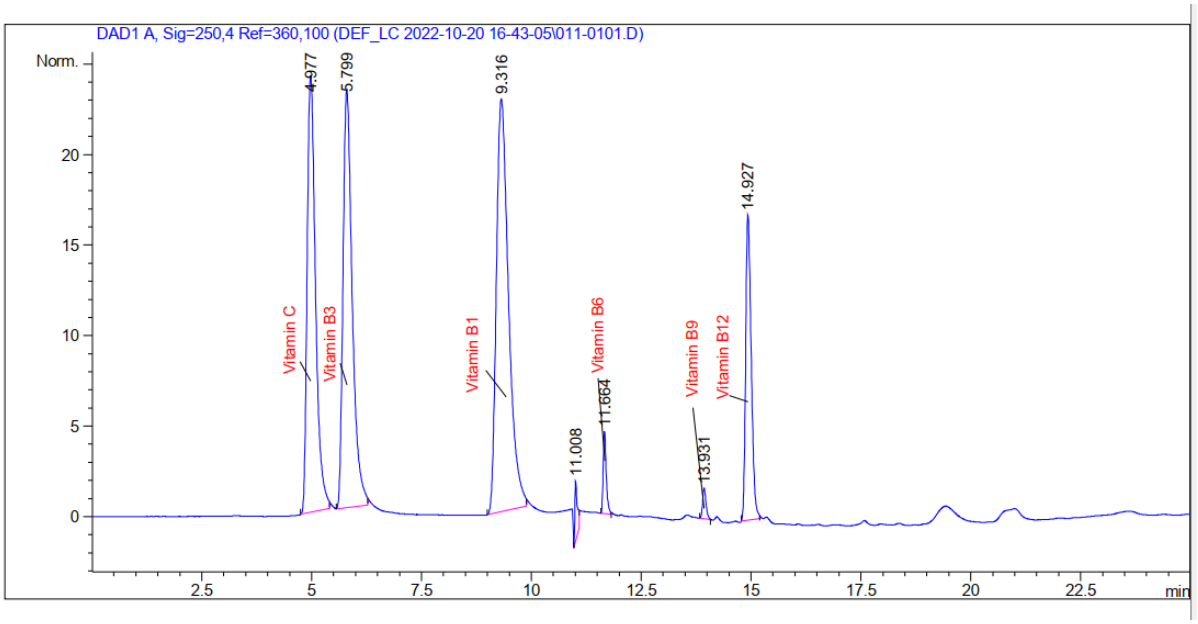


Figure 1. HPLC-DAD chromatogram of the water-soluble vitamin standard solution.

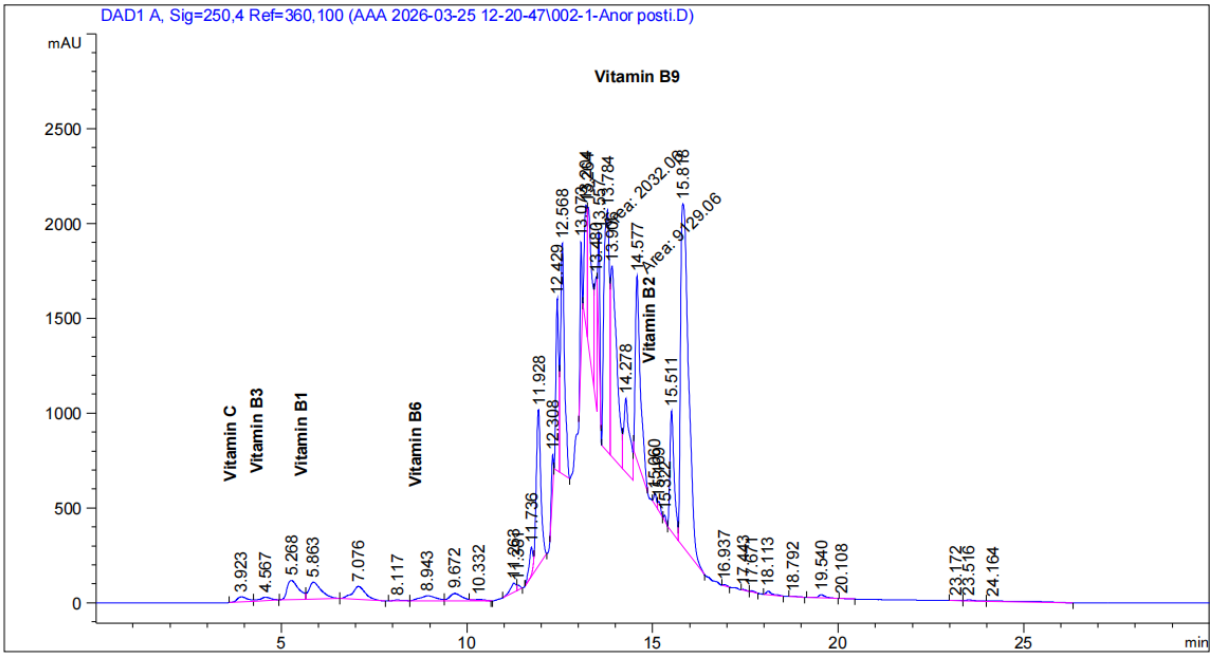


Figure 2. HPLC-DAD chromatogram of water-soluble vitamins in the pomegranate-peel extract test solution.

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3.2 Free amino-acid profile

Twenty free amino acids were reported, with a combined concentration of 14.456 mg/g (Table 3). Aspartic acid was the predominant analyte at 3.184 mg/g and accounted for approximately 22.03% of the reported free-amino-acid pool. Histidine was second at 1.735 mg/g (12.00%), followed by tyrosine at 1.495 mg/g (10.34%), leucine at 1.390 mg/g (9.62%), alanine at 1.346 mg/g (9.31%), and methionine at 1.253 mg/g (8.67%). Together, these six amino acids represented approximately 71.97% of the total reported free amino acids.

The remaining amino acids were present at concentrations below 0.6 mg/g. Cysteine (0.577 mg/g), glutamic acid (0.508 mg/g), valine (0.484 mg/g), asparagine (0.387 mg/g), glycine (0.371 mg/g), and serine (0.300 mg/g) formed an intermediate group. Isoleucine, arginine, proline, threonine, glutamine, tryptophan, phenylalanine, and lysine were present at lower levels. Lysine was the least abundant reported amino acid at 0.067 mg/g. The standard amino-acid chromatogram and extract chromatogram are presented in Figures 3 and 4, respectively.

Table 3. Free amino acids quantified in the dry pomegranate-peel extract

Amino acid	Mean concentration (mg/g)	Proportion of total (%)
Aspartic acid	3.184	22.03
Glutamic acid	0.508	3.51
Serine	0.300	2.08
Glycine	0.371	2.57
Asparagine	0.387	2.68
Glutamine	0.188	1.30
Cysteine	0.577	3.99
Threonine	0.190	1.31
Arginine	0.237	1.64
Alanine	1.346	9.31
Proline	0.211	1.46
Tyrosine	1.495	10.34
Valine	0.484	3.35
Methionine	1.253	8.67
Histidine	1.735	12.00
Isoleucine	0.245	1.69
Leucine	1.390	9.62
Tryptophan	0.172	1.19
Phenylalanine	0.116	0.80
Lysine	0.067	0.46
Total	**14.456**	**100.00**

Note. *The original table contained inconsistent units. The corrected unit is mg/g because the individual values sum to the reported total of 14.456 mg/g and the source narrative also used mg/g.*

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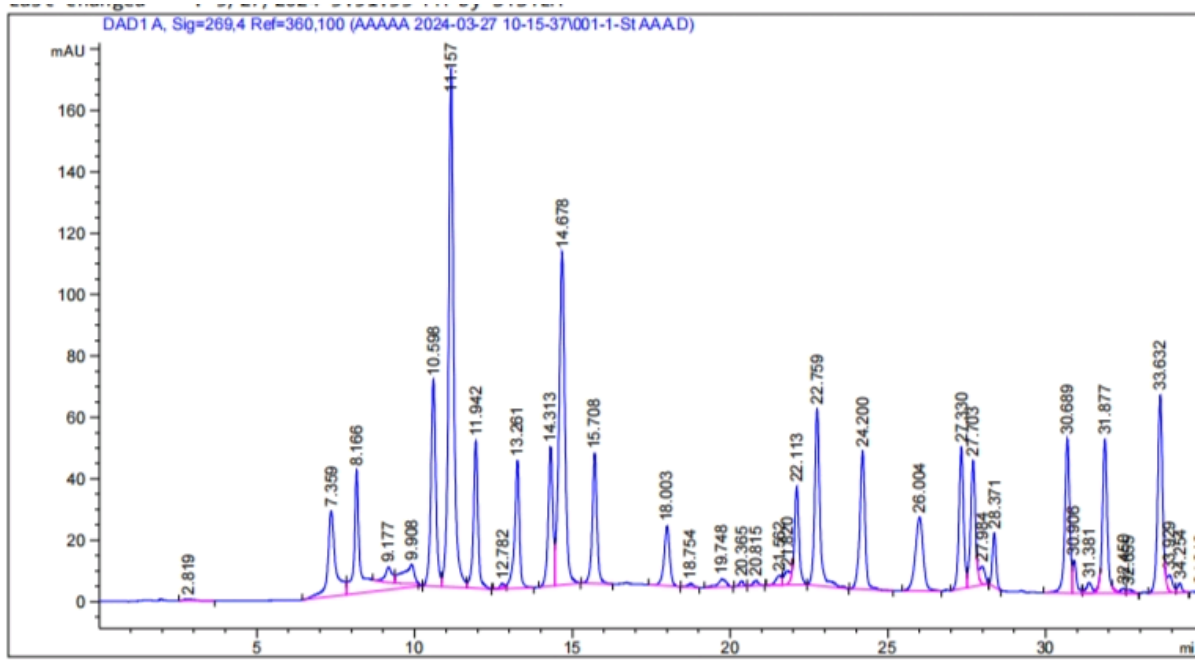


Figure 3. HPLC-DAD chromatogram of the amino-acid standard solution after PITC derivatization.

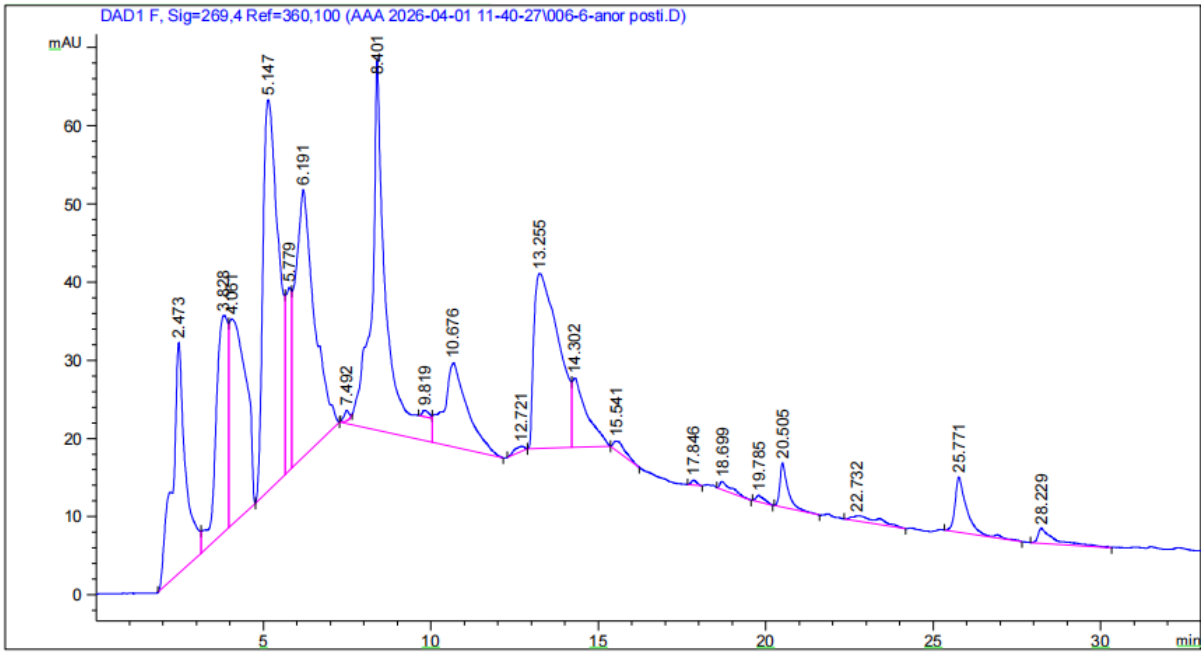


Figure 4. HPLC-DAD chromatogram of free amino acids in the pomegranate-peel extract test solution after PITC derivatization.

4. Discussion

The present analysis extends the chemical description of pomegranate peel beyond the phenolic compounds that dominate the literature. Earlier work has shown that pomegranate peel is chemically distinct from the edible aril and can contain a particularly rich spectrum of polar constituents [8-11]. The current findings indicate that an aqueous peel extract also contains measurable quantities of selected water-soluble vitamins and a broad range of free amino acids. These classes of compounds may be useful as supplementary markers when the extract is characterized across manufacturing batches.

Among the vitamins, folic acid was the highest measured analyte. This result is potentially important from a compositional standpoint, but it should be interpreted cautiously. Folic acid is normally present at much lower concentrations than major bulk constituents, and its determination in complex plant matrices is sensitive to extraction conditions, light exposure, oxidation, pH, co-eluting compounds, and the chemical form used as a standard. The chromatographic pattern in Figure 2 shows substantial matrix complexity around the labelled vitamin region. Therefore, confirmation by peak-purity analysis, matrix-

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matched calibration, standard addition, or an orthogonal detector would strengthen the assignment. The same caution applies to ascorbic acid, which is particularly susceptible to oxidation during sample preparation and storage.

The vitamin pattern also illustrates why compositional results should be distinguished from nutritional or therapeutic claims. The measured concentrations demonstrate the presence of the analytes in the tested extract, but they do not by themselves establish bioavailability, clinical efficacy, or a recommended dose. For pharmaceutical standardization, the immediate value of these data is analytical: they provide candidate quantitative attributes that may help distinguish a conforming extract from one that has undergone excessive degradation, dilution, substitution, or uncontrolled processing.

The free-amino-acid profile was dominated by aspartic acid, histidine, tyrosine, leucine, alanine, and methionine. The predominance of aspartic acid and the presence of both acidic, neutral, aromatic, sulfur-containing, and branched-chain amino acids indicate a chemically diverse free-amino-acid pool. Because the method targeted free amino acids after precipitation of proteins and peptides, the values should not be interpreted as the total amino-acid composition of the peel or extract. Total amino-acid analysis would require controlled hydrolysis of proteins and separate validation for amino acids that are unstable or transformed during hydrolysis.

The PITC derivatization approach used here is a well-established strategy for ultraviolet detection of amino acids [14,15]. Nevertheless, derivatization-based methods require careful control of reaction time, reagent composition, pH, evaporation, derivative stability, and injection timing. The complex baseline and partially unresolved regions in the test-solution chromatogram suggest that matrix effects and co-elution should be evaluated. For a validated assay, representative blank solutions, reagent blanks, spiked samples, duplicate preparations, system-suitability criteria, and resolution requirements should be specified.

The arithmetic consistency of the amino-acid data supports correction of the unit error in the original table. The individual values sum exactly to 14.456, matching the stated total. The narrative description also reported the values in mg/g. Accordingly, the amino-acid concentrations are presented throughout this revised manuscript in mg/g rather than μg/g. The original spelling “Lycine” has been corrected to leucine because lysine was separately listed at 0.067 mg/g, and the reported 1.390 mg/g value was described as leucine in the results narrative. Typographical errors in tryptophan and phenylalanine were also corrected.

From a standardization perspective, the strongest next step would be a multi-batch study. At least three independently manufactured batches should be tested to establish the natural and process-related variation of the vitamin and amino-acid profiles. Acceptance ranges should be based on observed batch data rather than on a single preparation. In parallel, one or more characteristic pomegranate markers—such as validated phenolic markers or a chromatographic fingerprint—should be incorporated because vitamins and amino acids are not unique to pomegranate peel. WHO guidance emphasizes that marker selection should reflect the intended function of the marker and should be integrated with identity, purity, and manufacturing controls [2].

A complete quality specification would also include botanical authentication, macroscopic or microscopic identity where applicable, moisture or loss on drying, ash values, extractable matter, residual solvents where relevant, microbial limits, pesticide residues, elemental impurities, mycotoxins, and stability-indicating assays [1-3]. Analytical procedures intended for routine release testing should be validated according to the relevant characteristics described in ICH Q2(R2), including specificity or selectivity, accuracy, precision, linearity, range, and robustness [4]. Because the available source record did not include these validation datasets, the current work should be regarded as an initial compositional characterization rather than a completed pharmacopoeial standard.

Despite these limitations, the study provides a useful quantitative starting point. The vitamin table, amino-acid table, and four chromatograms create a more informative chemical record than a general claim

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that pomegranate peel is “rich in biologically active compounds.” The results identify specific analytes, report their concentrations, and reveal which compounds dominate the measured profiles. With verification across batches and full analytical validation, these data could contribute to a layered standardization framework combining botanical identity, chromatographic fingerprinting, selected marker assays, and general quality tests.

5. Study Limitations

The principal limitation is that the available source document contained only summarized concentration values and chromatographic images. It did not include raw chromatographic data files, integration reports, calibration curves, certified-standard details, replicate-level measurements, standard deviations, recovery experiments, system-suitability criteria, or formal validation results. These elements cannot be reconstructed through proofreading and were therefore not invented in this revision.

The extract was described as an aqueous dry extract, but the source record did not provide full botanical and manufacturing traceability. Results from one incompletely documented extract batch cannot establish universal composition or pharmacopoeial limits for pomegranate peel. Future work should examine authenticated material and independently manufactured batches, and should report all extraction and storage parameters.

The measured vitamin and free-amino-acid concentrations provide compositional information only. No biological assay, toxicity test, stability study, bioavailability assessment, or clinical investigation was included. Consequently, the present data should not be used to make therapeutic claims.

6. Conclusion

HPLC-DAD analysis demonstrated that the tested dry aqueous extract of pomegranate peel contained six quantified water-soluble vitamins and 20 reported free amino acids. Folic acid was the highest measured vitamin at 98.84 µg/g, while aspartic acid was the predominant free amino acid at 3.184 mg/g. The total free-amino-acid concentration was 14.456 mg/g. The results support the use of vitamin and free-amino-acid profiles as supplementary compositional attributes for pomegranate-peel extract. They do not, however, constitute complete pharmaceutical standardization. Botanical traceability, independently produced batches, validated calibration and recovery data, precision and robustness studies, stability testing, contaminant control, and characteristic pomegranate marker assays are still required. The revised data tables and chromatographic figures provide a clear foundation for those subsequent studies.

Declarations

Funding: Not reported in the source document.

Conflict of interest: Not reported in the source document.

Data availability: The chromatographic figures and summarized concentration data are included in this manuscript. Raw chromatographic files and replicate-level data were not supplied with the source document.

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