INTRODUCTION

Papain [EC 3.4.22.2] from the Carica papaya L. plant is an endopeptidase belonging to the cysteine endopeptidase family [1]. It was the first enzyme of this family isolated and characterized from the latex of leaves and fruits of C. papaya [2]. It is obtained by making incisions on the epicarp of unripe papaya [3]. Commonly, more active papain can also be obtained from greener or unripe fruit [4]. Because of their proteolytic activity, they are of vital importance in biological processes in living organisms [5, 6]. Papain is commonly used in various textiles [7], pharmaceutics [8], and food industries [9]. Papain has been recognized for the treatment of trauma, allergies and injuries [10, 11]. Likewise, papain also acts as a chemical dentin excavation [12], antifungal [13], and anti-inflammatory [14] and to treat several types of cancer like Colorectal, Lung and Prostate cancers [15-17]. SANCO guidelines were employed for the LC-MS/MS experimental planning. The advantage of these guidelines is three or more concentration matrix factors can be analyzed simultaneously [18]. SANCO guidelines generally provide matrix-matched calibration which helps to develop the quality analytical method and validation measures for pesticide residue analysis in food and feed [19].

Since matrix effects are known to frequently occur in both gas chromatography and liquid chromatography techniques, they should be evaluated at the first step of method validation. Matrix effects are often addressed through matrix-matched calibration. Calibration should be performed using blank matrix extracts, particularly of the same kind as the sample. In comparison with chromatographic interferences brought on by overlapping or unresolved peaks from co-extracted substances, this approach is intended to make up for matrix effects and recovery losses. For comprehensive guidance on these procedures, the SANCO guidelines can be referenced [20].

According to the literature review, a few analytical techniques for estimating papain in bulk and pharmaceutical solid dosage forms were described by ultraviolet spectrophotometry method [21, 22]. For the qualitative and quantitative examination of papain and its formulations, high-performance liquid chromatography was used [23, 24]. The reported method explains the use of U/HPLC, which was not sensitive enough to identify small variations in papain and its stability-related issues. The validation of analytical techniques was not demonstrated completely in a clear manner, using statistical models that develop the interest to develop the method that is suitable for its usage.

As a result, the main aim of this project work was to develop and validate a standard method for detecting papain by LC/M-MS technique, which might aid in understanding its therapeutic role. There are presently inadequate ways for identifying Papain and its biological matrices concurrently utilizing the LC-MS/MS methodology. In the present work, the SANCO guidelines matrix calibration approach was attempted with different approaches for the analysis of the enzyme. The study’s purpose was to successfully develop and verify the LC-MS/MS method for papain and its supplementary sources, such as papaya stem and leaf with matrix interferences. The validated technique exhibits acceptable sensitivity and selectivity in a suitable Chromatographic run time.

MATERIALS AND METHODS

Chemicals and standards

Analytical grade active pharmaceutical ingredients (API) were employed for method development and validation. All APIs came with a certificate of analysis indicating that they fulfilled product specifications and had a purity of more than 99%.
The reference standard of Papain was purchased from Antozyme Biotech Pvt. Ltd, Gujarat. LCMS/MS grade Formic acid and acetonitrile have been purchased from SD Fine Chemicals. Water for mobile phases and sample preparation was produced in-house using a Milli-Q RO system for instrument water purification and using it was filtered by 0.22 µm polyvinylidene fluoride (PVDF). All other chemicals were of analytical quality and obtained commercially.

Instrument

Perkin Elmer Qsight LX-50 UHPLC equipment was used for method development and validation with electrospray ionization interface, with programmable injection volume from 0.1-100 µl loop (depending upon mode and loop). A QsightTM LX-50 Autosampler, QsightTM LX-50 Oven, QsightTM LX-50 Pump, and Qsight 420 Mass spectrometer, and for system control, data acquisition, and integration, SIMPLICITY3Q data station was utilized.

Mass spectrometer and chromatographic conditions

The analytes were separated chromatographically on a 30 °C Quasar C18 column with dimensions of 150×2.1 mm and particle size of 3 µm. A gradient elution mode was employed with a mobile phase A consisting of 0.2% formic acid in water and mobile phase B consisting of formic acid in acetonitrile with the ratio of 98:2% with a flow of 0.5 ml/min with an injection volume of 10 µl. Table 1 displays the gradient mode programming parameters.

### Table 1: Parameters of gradient mode

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Time (Min)</th>
<th>Flow (ml/min)</th>
<th>Mobile phase A</th>
<th>Mobile phase B</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>0.00</td>
<td>0.400</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>02</td>
<td>1.00</td>
<td>0.400</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>03</td>
<td>1.01</td>
<td>0.400</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>04</td>
<td>2.00</td>
<td>0.400</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>05</td>
<td>4.00</td>
<td>0.500</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>06</td>
<td>5.00</td>
<td>0.500</td>
<td>95</td>
<td>5</td>
</tr>
</tbody>
</table>

The estimate was performed in positive ion modes with a QSIGHT 420 Mass spectrometer fully equipped with an Electron Ionization ionization source. The temperature and voltage of the ion spray were set at 200 °C and 5500 volts, respectively. The nebulizer gas, drying gas, and curtain gas pressures were set to 35, 45, and 25 psi, respectively. With a dwell period of 97.00 units, Papain’s declustering potential, entry potential, collision energy, and collision exit potential were 50, 24, 19, and 13V, respectively. Multiple reactions monitoring (MRM) mode was used for ion detection, with mass transitions of m/z 360→522 for the Papain internal standard.

Preparation of standard papain stock and working solution

A total of 10 mg of papain was dissolved in 10 ml of water (1 mg/ml). A working concentration of 1 mg/ml was obtained from the prior mentioned stock solution. Furthermore, a calibration standard that ranges from 1 to 2000 ng/ml of papain was developed from the working standard.

Sample preparation

5 gm of shadow air-dried C. papaya leaf and stem were taken and crushed with mortar and pestle. The coarse powder was soaked in 15 ml of LC grade water, vortexed for 1 min and sonicated for 30 min at 24 °C. This extraction was carried out several times with the same material. The resultant solution was sonicated and centrifuged in a cold-refrigerated centrifuge at 10,000rpm for 10 min at 4 °C. The samples were then filtered using a 0.22 µm pore size polycarbonate membrane. To assess the matrix effect, the control sample preparation was handled in the same manner and stored at 5 °C in refrigerator condition [25].

Optimization of the mass range

Standard Papain (1000 ng/ml) was used for mass analysis. The precursor ion at m/z 522.0 and production at m/z 360.3 have been obtained to monitor papain concentration.

Method validation

The new analytical technique was verified in compliance with ICH guidelines Q2 (R1) utilizing many validation criteria such as specificity, linearity range, accuracy and precision, limit of detection (LOD) and limit of quantification (LOQ), matrix effects, robustness and system suitability [26].

The specificity was determined by comparing the chromatograms of two C. papaya plant leaf and stem matrices to a standard. The linearity was tested with five different papain concentrations ranging from 1 to 2,000 ng/ml. Statistical analysis was utilized to calculate the correlation coefficient (R²), slope (m), and intercept values. The detection and quantification limits were calculated using the signal-to-noise ratio. The calibration range was used to choose the quality control standards for papain, which were LOQ: 350 ng/ml, MQC: 1000 ng/ml, and HQC: 1800 ng/ml. To check that data is correct within the defined range, standard deviation, percent RSD, and standard error of mean were also assessed. Three different Papain concentrations (LOQ: 350 ng/ml MQC: 1000 ng/ml, HQC: 1800 ng/ml) were tested at different times on the same day to assess intra-day precision, followed by a second day of repetition to determine inter-day or intermediate precision. By comparing the retention time (Rt) and MS signal intensity, the matrix effect was determined by a typical blank matrix spiked with a predetermined amount of papain standard to those obtained.

To validate the matrix match between papaya leaf and stem, a similar comparison was performed using both matrix samples spiked with the same Papain standard. Flow rate, column temperature, wavelength detection, injection volume, mobile phase and pH were all used to test the robustness of the chromatography technology. Short-term solution stability was tested for both the working solution and the internal standard.

RESULTS AND DISCUSSION

Chromatography and tandem mass spectrometry

The novel separation method was developed to prove suitable chromatographic performance while also being easily adaptable on regular LCMS/MS equipment. In this work, chromatographic and mass parameters were extensively optimized for the analytes and internal standard determination. To the best of our knowledge, chromatographic conditions, particularly mobile phase comparison, played a critical role in developing good chromatographic behavior, which includes peak symmetry sensitivity, short run duration, and suitable ionization [27-29].

For the optimization of the ESI setting for papain standard, Quadrupole full scans were done in a positive ion detection mode. Throughout direct infusion experimentation, the mass spectra for papain revealed peaks at m/z 145.3, 163 and 325 as protonated molecular ions generated by direct photoionization. The MRM transitions m/z 360→145.3, 360→163, 522→325, 522→163.1 were employed for the measurement of Papain after extensive optimization of mass spectrometry settings. The mass spectra fragmentation pattern of precursor and product ion of the Papain enzyme is shown in fig. 1.
The LC-MS/MS protocol’s optimized chromatographic conditions were aimed at achieving identifiable symmetrical peaks with acceptable resolution. The C18 column with 3 µm particle size ran at a speed of no more than 0.25 ml/min with gradient elution mode consisting of mobile phase A as 0.2% formic acid in water and B as 0.2% formic acid in acetonitrile, demonstrating the best results for LC separation as well as providing the best conditions for MS/MS measurements.

**Method validation**

**Specificity**

Representation of the chromatograms indicates the absence of interference peaks in papain standard at the analytes retention times. The retention time of the Papain standard was 0.84 min. Therefore, the absence of interference peaks from endogenous substances supports to be the suggested method is selective and highly sensitive (fig 2).
The linearity with five unique concentrations was developed for values ranging from 1 to 2000 ng/ml. $R^2$ (correlation coefficient) was calculated to be 0.998. The calibration curve findings' standard deviations (SDs) were determined to be within the limits of the regression equation of $y=13840x+1820.9$ (fig. 3).

**LOD and LOQ**

The LOD for Papain was determined to be 170.5ng/ml with a signal-to-noise ratio of 3:1. The LOQ for Papain was determined to be 516.8ng/ml with a signal-to-noise ratio of 10:1. Reagents and the use of non-LC-MS/MS grade solvents can change these values, resulting in signal-to-noise ratio changes. As a result of this discovery, the developed method has a high sensitivity.

**Accuracy and precision**

For accuracy testing, quality control levels of low (LQC), medium (MQC), and high (HQC) were established. The procedure's accuracy was assessed by calculating the mean % recovery for three QC samples. For precision experiments, recovery ranged from 97% to 99%, with percentage RSD ranging from 0.15% to 1.56% (table 2).

**Matrix effect**

For the calibration of *C. papaya* stem and leaf extracts, the matrix-induced ionization method was used, which is already been demonstrated [30] that SANCO guide’s matrix-matched calibration approach was selected as the most reliable for quantification analysis.

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Hence papain extracted from *C. papaya* leaves and stems was chosen as a matched matrix source. *C. papaya* is a plant with a higher concentration of papain. There was no difference in peak quality or size between the blanks. When compared to the standard, the unspiked extract of *C. papaya* showed no changes in peak form [31]. Both matrices, stem and leaf, were compared using standard addition calibration curves constructed by spiking those extracts with known doses of Papain and using four copies for each concentration value to evaluate if signal strength altered in *C. papaya* samples [32].

Although the calibration curves were not equal due to the presence of Papain in the *C. papaya* samples, the slopes did not alter significantly.
This verified the following use of *C. papaya* extracts as a matching matrix. The lack of relevance of the matrix on signal quality in our investigations compared to other laboratories might be explained by variations in analyzed species extract composition, technique specifications, equipment utilized in experiments, sample preparation, or a combination of variables [33].

**Robustness**

The method’s robustness was investigated by changing the experimental settings. When the experimental conditions, such as the operators, reagent supply, column of the same type, and optimized conditions, were changed. However, no substantial changes in chromatographic parameters were detected [34]. To examine the method’s robustness, the flow rate (0.2, 0.4, and 0.5 ml/min), pH (4.5, 5.5, and 6.5), and formic acid percentage (50%, 85%, and 95%) were all measured. Table 3 shows that the % RSD for the flow rate, pH, and formic acid percentage were computed and found to be within the limit, indicating that the established technique was determined to be resilient [35].

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Adopted conditions</th>
<th>Retention time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow rate (ml)</td>
<td>0.2</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>0.91</td>
</tr>
<tr>
<td>pH</td>
<td>5.5</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>6.0</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>6.5</td>
<td>0.85</td>
</tr>
<tr>
<td>Mobile phase ratio</td>
<td>50</td>
<td>0.89</td>
</tr>
<tr>
<td>(Formic acid %)</td>
<td>85</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>95</td>
<td>0.88</td>
</tr>
</tbody>
</table>

**Solution stability**

After roughly 24 h at refrigerated temperatures ranging from 2 to 8 degrees Celsius, papain at the working calibration standard level and internal standard solution at the working internal standard solution level were stable. The Papain and internal standard solution maintained at room temperature were only stable for 12 h, demonstrating a significant shift in activity and peak area after being introduced into the system.

**CONCLUSION**

In conclusion, our original work has successfully established the LC-MS/MS method, enabling the simultaneous quantification of Papain standard and Papain within biological matrices, specifically papaya leaves and stems. This achievement marks a significant milestone in facilitating accurate determination and analysis of Papain, offering a robust approach for future studies in this domain. The Quasar C. column was discovered to be the best option for attaining satisfactory chromatographic resolution in a gradient mode at 0.5 ml/min using 0.2% formic acid in water and 0.2% formic acid in acetonitrile (98:2%). Because the analytes have diverse physicochemical properties, method development was meticulously developed to allow the analysis of the analyte in a single test process. An accurate, easy, precise, highly sensitive, specific, and cost-effective approach for quantifying Papain and its matrix interferences was effectively developed and utilized. Under the parameters of this study, the method proposed in this paper is adequate for reliable assessments of papain content in extracted samples and reference papain. The technique performed brilliantly in terms of selectivity, linearity, accuracy, precision, recovery, detection limit, and quantitation limit. Furthermore, in terms of analytical run time, the novel procedure is quicker than previously published approaches. Because of its great sensitivity, the newly developed and validated approach will be valuable for clinical pharmacokinetic, biopharmaceutical, and bioequivalence research.

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**AUTHORS CONTRIBUTIONS**

All the authors contributed significantly to this manuscript; Chandan C has conceptualized and Writing—the original draft of the manuscript. Rushilshekh Shraji Agalave and Tanya Tyagi have done data acquisition, analysis and data interpretation by Aditya Kumar Singh. Ramesh J has done review writing and editing of the manuscript. English grammar and language checking. The critical revision of the manuscript was done by Dr. Sushma B V, technical and material support is being provided by Dr. Phani Kumar G and the supervision and final approval was given by Dr. Jayaprakash M R.

**CONFLICT OF INTERESTS**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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