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Study of quality control and uncertainty in estimation of capsaicinoids content and pungency in real chili samples using RP – HPLC

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Abstract

A method was developed and validated for the determination of capsaicin in real chili samples collected from the market by reverse phase HPLC. The recovery data were obtained by spiking blank samples of reagent methanol with capsaicin at concentration levels of 10.0 µg/ml, yielding recoveries in the range of 90 – 110%. Precision values expressed as relative standard deviation (RSD) were in the range of 1.62 – 13.18%. Linearity was studied in the range 10 – 200 µg/ml and the coefficient of correlation was higher than 0.98. for all capsacinoid compounds. Method Detection Limits (MDLs) and Limits of Quantification (LOQs) were established. The overall uncertainty of the method was estimated. About 20 chili samples collected from the market were analysed for capsaicin content. The pungency of these samples expressed in SHU units were also determined. According to the validation data and performance characteristics as well as the high sample throughput, the proposed method is suitable for routine application. The capsaicinoids content and Pungency of the samples were between 0.17 – 0.82% and 25500 -124290 SHU, respectively.

Introduction

Chilies are one of India's major export commodities. Chili comes in a wide variety of shapes, sizes, colours and in different degrees of pungency. India is the only country rich in many varieties with different quality factors. Products are also available as powder and oleoresins. Indian chili is exported to many countries. Chilies are generally known as ripen fruits of various species of genus capsicum. They play an important role as one of the most commercial crops used both as condiment or culinary supplement and as vegetable. Commonly, their hot sensory taste is due to capsaicinoids as the major group of organic compounds which is closely related to the family of alkaloids, and are known to be biosynthesized and accumulated in the placenta of Capsicum fruits. The major capsaicinoids present in most varieties of the chili are capsaicin (tran-8-methyl-N-vanillyl-6-nonenamide)and dihydrocapsaicin (8-methyl-N-vanillylnonanamide). In addition, other minor ones are also found such as nordihydrocapsaicin, and dihydrocapsaicin. Capsaicin has been used in neurological research to stimulate sensory nerves and also to treat bladder inflammation. It is also found in topical ointments used for arthritis and neuralgia (Kaale *et al*., 2002), and exerts its effect on the sensory nerves by interacting with the vanilloid receptor, promoting the release of substance P as well as other cytokines (Surh *et al*., 2005).The determination of capsaicinoids in chili peppers, topical cream (Kaale *et al.*, 2002), self-defense weapons (Reilly *et al*., 2001a) and aerosol defense sprays (Spicer and Almirall, 2005) has been of increasing interest for many reasons.

Chilli pungency is measured in Scoville Heat Units (SHU) and Scoville organoleptic test was used initially for measuring SHU (Scoville, 1912). However, high-performance liquid chromatography (HPLC) method has replaced the organoleptic method since the HPLC method is considered the most reliable and accurate method for determining both the amount of capsaicin and pungency in chili samples. HPLC is currently the most popular and reliable technique for the analysis of capsaicinoids. The technique has been mainly associated with UV absorption detection. Reversed phase-HPLC separation of capsaicinoids in some chili varieties was achieved using UV-Visible detector method. The linearity range of calibration curve was 0.0 to 200 ug/ml Validation was carried out by establishing the repeatability and Limits of detection (LOD/MDL) and Limit of quantitation (LOQ). The optimized conditions were applied for the determination of capsaicinoids in varieties of the chili samples.

Considering the consumer's health and safety it has become imperative to ensure that the spices available in the market for sale is safe and free from harmful substances. Stringent limits have been considered in the Prevention of Food Adulteration

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Act, 1954 and subsequent amendments in Act. The Rules framed there under regarding the presence of capsaicin in samples samples require development and quality control of analytical method for accreditation by internationally established organization such as NABL under ISO/IEC 17025. The paper reports the study carried out for development of quality control in the analysis of capsaicin content in chilli samples. Methods to determine capsaicin include the extraction of the analytes from the matrix, appropriate cleanup of the raw extracts and subsequent determination by liquid chromatography (LC). Globalization of commodities market and concerns for the consumer has put pressure on regulatory agencies to increase capsaicin monitoring programs in terms of specificity of analysis and number of samples analyzed. These demands have caused the development of methods to reliably and rapidly detect as many capsaicinoids as possible from a single extraction.

In this work, reverse phase HPLC was used. The application of HPLC methods allows one to obtain a sensitivity and selectivity gain over other conventional methods. The aim of this study was to develop and validate an LC method for determining capsaicin in chilli samples determined in less than 20 min after extraction.

Materials and Methods

Capsaicin reference standards were obtained from HiMEDIA, India. Chromatography grade solvents (acetonitrile, methanol) were purchased from Merck, India. Purified RO grade water with a conductivity of 0.5 µSi/cm was utilized during the analysis. A Thermo make LC chromatograph (Thermo Fisher Scientific Instruments, San Jose, CA95134, and USA) was used. A Thermo Fisher Scientific Dionex grade 3000 Ultimate UHPLC was coupled to a Dionex multisolvent delivery system, in-line degasser AF, auto sampler, a Rheodyne injector with sample loop of 20 µL, and a Dionex variable wavelength UV-Vis detector. A Hypersil BDS Phenyl C18 column (4.6 mm i.d. × 250 mm, 5 µm particle diameter) was used.

The optimization for HPLC analysis of capsaicinoids was investigated by varying the composition of mobile phase whereas the other conditions used throughout the experiment were as follows: flow rate of 1.0 ml/min, uv-vis detector at 225nm. Binary solvent mixtures of 40-60% v/v of acetonitrile and 1g/l of phosphoric acid solution were used as a mobile phase. The mobile phase was filtered through a 0.45 µm nylon membrane and degassed before use. The column was equilibrated with the mobile phase for 30 min or until a steady detector baseline was achieved. Ten ul of sample was injected into the injector.

The chilli samples were obtained from the local market. About 2.5 g of the powdered sample was taken in a beaker. 8o ml of methanol was added and the flask was allowed to macerate for 30 min. The flask was then placed in ultrasonic bath for 15 min. The contents were transferred to a 100 ml volumetric flask. The remaining contents in the beaker were rinsed with methanol and made to 100 ml with methanol.

Stock standard solution was (200 µg/ml) prepared from pure certified standard reference material by accurately weighing the pure material on a 5 decimal place analytical balance. The material was dissolved in methanol and volume made up to 10 ml in certified volumetric flask. The standards were stored at low temperature in a freezer. The calibration standard were at five concentration levels for each compound by adding appropriate volume of one or more stock standards to a volumetric flask and diluting to volume with methanol. While preparing working standards, a record was kept of the identity and amount of all solutions and solvents employed. The standards were labeled indelibly, allocated an expiry date, and stored at low temperature in the dark in containers that prevent any loss of solvent and entry of water.

In order to carry out the quantitative analysis of the samples with LC reverse phase mode was used due to its applicability for all the capsaicin compounds. The auto injection, flow rate and wavelength conditions were fixed for the analysis. Identification and confirmation of the target compounds was based on the use of retention time of the chromatographic peak of the analyte. The RTs were established for all the capsaicinoids under study.

Results and Discussion

In order to carry out capsaicinoids analysis it was necessary to develop an in-house quality control program for ongoing analysis of spiked samples. Ongoing data quality checks were compared with established performance criteria to meet the performance characteristics of the method. The analysis of capsaicin in samples requires validation of all procedures (steps) that were undertaken in the method. This required assessment of linearity, recovery (as a measure of trueness or bias) and precision.

Linearity was studied in the range 10–200 µg/ml with seven calibration points by matrix-matched standard calibration. Calibration curves for all the

Table 1. Concentration of Capsaicinoids in real chili pepper samples

Samples	Total Capsaicinoids, %	SHU
Sample 1	0.77	115733
Sample 2	0.76	117098
Sample 3	0.78	117090
Sample 4	0.87	130498
Sample 5	0.27	40500
Sample 6	0.17	25500
Sample 7	0.18	26903
Sample 8	0.29	43050
Sample 9	0.29	44640
Sample 10	0.28	42660
Sample 11	0.35	52650
Sample 12	0.37	56385
Sample 13	0.36	53940
Sample 14	0.49	74235
Sample 15	0.82	124290

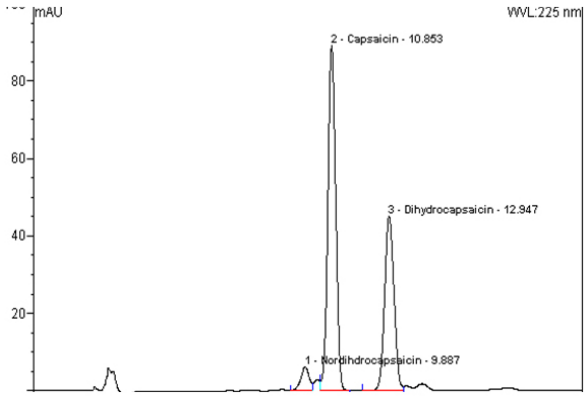

Figure 1. Chromatogram for Capsaicinoids in standard

capsaicinoids were developed in the 10–200 µg/ml range. Figure 1 illustrates the chromatogram for capsaicinoids in standard. Linear calibration graphs were constructed by least-squares regression of concentration versus relative peak area of the calibration standards. Linearity values, calculated as determination of correlation coefficient (r^2), were in the range 0.9814–0.9999. Figures 2-4 summarises the calibration curves for (1) nordihydrocapsaicin, (2) Capsaicin and (3) Dihydrocapsaicin.

Accuracy was evaluated in terms of recovery by spiking blank samples of media with the corresponding volume of the capsaicin working standard solution. Total of seven samples, one on each day, were spiked with a concentration of 10.0 µg/ml. The samples were than processed for analysis by HPLC. The results of day to day analyses are summarized in Table 2. Recoveries between 99 and 100% were found in samples. The minimum RSD was 1.62 (Dihydrocapsaicin) and the maximum 13.18 (Nordihydrocapsaicin). Therefore, these results meet the requirement criteria of trueness or mean recovery for quality control. Method detection limits (MDL) were also determined for all the capsaicinoids under this study. It provides a useful mechanism for

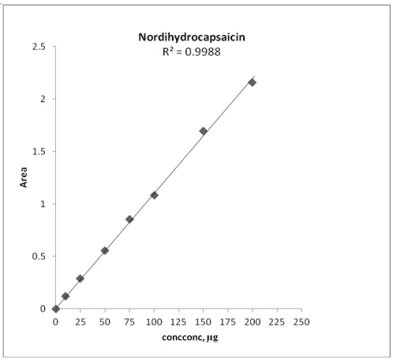

Figure 2. Calibration curve for Nordihydrocapsaicin

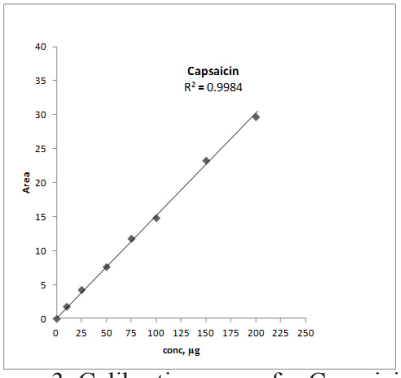

Figure 3. Calibration curve for Capsaicin

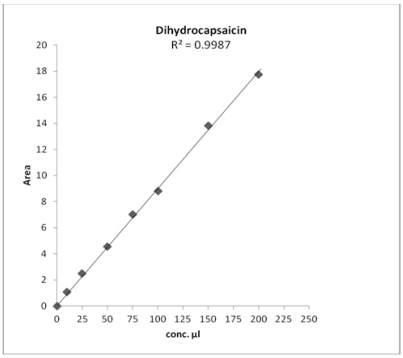

Figure 4. Calibration curve for Dihydrocapsaicin

illustrating the capability of the analytical method. MDLs were calculated for the capsaicin as follows:

The sample standard deviation is multiplied by the correct Student's t-value from the statistical Tables (Kelly *et al*., 1992). In the present study seven replicates were taken, hence six degrees of freedom was considered. t is found to be 3.143. The MDL was calculated for a compound like capsaicin as follows: MDL = (s)(t-value) = 0.001775 x 3.143 = 0.005578 µg/ml. Rounding to the correct number of significant figures, the calculated MDL becomes 5.55x 10^{-3} µg/ml.

Similarly, LOQs were subsequently established as 10 times the Standard Deviation of the recovered pesticide. The limit of quantitation was also calculated as :

LOQ = 10 x (s) = 10 x 0.001775 = 0.01775 µg/ml

The MDL and LOQ were thus calculated for all the capsaicinoids under study and are summarized in

Table 2. Recovery study for Capsaicinoids in chili powder

Name of Capscinoids	RT	Test 1 µg/ml	Test 2 µg/ml	Test 3 µg/ml	Test 4 µg/ml	Test 5 µg/ml	Test 6 µg/ml	Test 7 µg/ml	SD	RSD	Mean	Recovery %	MDL µg/ml	LOQ µg/ml
Nordihydrocapsaicin	9.89	0.081	0.109	0.107	0.081	0.112	0.105	0.103	0.013	13.18	0.099	99.7	0.041	0.132
Capsaicin	10.86	0.097	0.100	0.100	0.098	0.102	0.099	0.100	0.001	1.77	0.100	100.0	0.005	0.017
Dihydrocapsaicin	12.97	0.098	0.098	0.101	0.098	0.099	0.101	0.101	0.001	1.62	0.099	99.0	0.005	0.016

Sample concentration = 0.10 ug/ml

Table 3. Values and Uncertainities in Capsaicin measurements

Description	Value x	u(x)	u(x)/x
Mass of the capsaicin(ug)	10	7.06×10^{-3}	7.06×10^{-4}
Purity	99.0	5.80×10^{-4}	5.80×10^{-5}
Volume of flask(mL)	10	2.52×10^{-2}	2.52×10^{-3}
Volume of flask(mL)	10	2.52×10^{-2}	2.52×10^{-3}
Volume of pipette(mL)	1	2.32×10^{-2}	2.32×10^{-2}
Volume of pipette(mL)	0.2	2.35×10^{-2}	1.17×10^{-1}
Coeff.correlaton	0.9980	3.29×10^{-3}	3.30×10^{-3}

Table 2.

Attempt was also made to estimate the uncertainty associated with the analytical method in methanol matrix by applying a bottom-up approach. All data appearing in this study complies with NABL 17025 requirements. It was implemented in our laboratory as a capsaicin analysis routine method and our laboratory was recently accredited. The uncertainty of each step was estimated identifying which of them are relevant in the global uncertainty. The values and uncertainties are shown in Table 3. The standard uncertainties associated with each step is quantified by estimating analyte concentration from the calibration curve, calculating recovery of the sample extract. After obtaining the standard uncertainty ($u(x)$), expressed as a standard deviation, and combined standard uncertainty were determined.

The different aspects explained above for estimating the combined uncertainties have been applied to the capsaicin analysis. Table 3 summarises the relevant information for calculating uncertainties associated with the preparation of primary standard solutions, volumetric materials, and analytical balance. In some cases, it is feasible to use relative uncertainties which represent the value of the uncertainty normalized. It is obtained as the quotient between the standard uncertainty $u(x)$ and the value of x:

$$Urel(x) = \frac{U(x)}{x} \quad \text{or} \quad urel(x) = \frac{u(x)}{x}$$

The uncertainty estimation was carried as per the following steps:

(1) *Specifying the measurand.* This involved making a clear statement of what is being measured, including the relationship between the measurand and the input quantities (measured quantities, constants and calibration standard values. (2) *Identifying uncertainty sources* i.e listing the possible sources of uncertainty, usually specified in the above step. (3) *Quantifying uncertainty components* i.e. estimating the uncertainty component associated with each potential source of uncertainty identified. The different contributions to the overall uncertainty is expressed as standard deviation which is calculated depending on the data available *from a standard deviation value* (this value is directly used); *from a coefficient of variation*; *from the standard deviation of experimental data sets*; *from a declared purity and uncertainty value* (which is given in a certificate of calibration for reference materials) and *from a correlation coefficient of calibration curves* etc. *Calculate combined uncertainty* by combining different contributions to the overall uncertainty according to the appropriate rules. The combined standard uncertainty $u(f)$ is calculated as

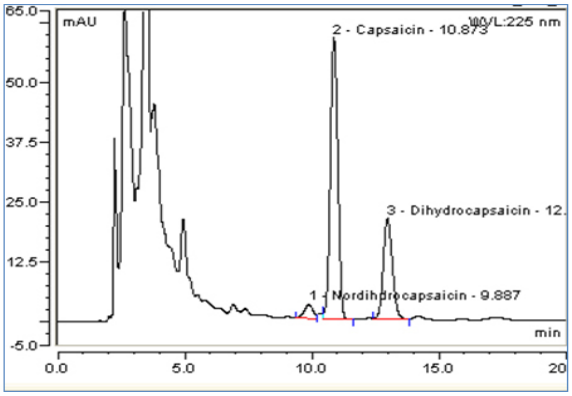

Figure 5. Chromatogram for chilli sample with Capsaicinoids

$$u(f) = [c^2(x)u^2(x) + c^2(y)u^2(y) + \cdots]^{1/2}$$

Where c is a sensitivity coefficient associated to each one of variables, given by the partial derivative of the function: $c(x) = \partial f/\partial x$ and (5) *Expanded uncertainty* by applying the appropriate coverage factor.

The combined uncertainty and expanded uncertainty were calculated for all the capsaicinoids under study.

For sake of illustration, calculation of uncertainty in analyte concentration for capsaicin is as below:

$$u_c(\text{c capsaicin}) = \text{c capsaicin}\{[\frac{u(P)}{P}] + [\frac{u(m)}{m}]^2 + [\frac{u(Vflask)}{Vflask}]^2 + [\frac{u(Vpip1)}{Vpip\,1}]^2 + [\frac{u(pip\,2)}{Vpip\,2}]^2 + [\frac{u(rep)}{rep}]^2 + [\frac{u(calib)}{calib}]^2 + [\frac{u(recov)}{recov}]^2\}^{1/2}$$

where u(P) is the uncertainty in purity of the capsaicin as quoted in the suppliers certificate, u(m)

is the uncertainty in the mass of the capsaicin in the certified reference standard solution, $u(V_{flask})$ is the uncertainty in volumetric measurements estimated by considering the influences of calibration, repeatability and temperature effects and determined by measurement of uncertainty in internal volumes and variation in filling volumetric flask to the mark, $u(v_{pip1})$ and $u(V_{pip2})$ are the uncertainties in volumetric measurements using pipettes , u(rep) is the uncertainty in repeatability estimation considering the standard deviation of replicate divided by $\sqrt{3}$.U(calib) is the uncertainty in calibration determined from the coefficient of correlation obtained for the calibration curve. U(recov) is the uncertainty in recovery of spiked samples involving precision and the homogeneity of the capsaicin sample. The precision was determined by measuring the standard deviation of a set of spiked samples, which were extracted and analysed each day.

$$u_c(c_{capsaicin}) = c_{capsaicin}\{33.64 \times 10^{-10} + 6.25 \times 10^{-8} + 5.29 \times 10^{-4} + 1.21 \times 10^{-4} + 16.16 \times 10^{-6} + 4.023 \times 10^{-3} + 4.147 \times 10^{-2}\}^{1/2}$$
$$= c\ capsaicin \times 0.2130$$

The expanded uncertainty $U(C_{capsaicin})$ was subsequently determined to develop an interval within which the value of the measurand may lie. A factor of 2 was thus used for obtaining a confidence level of 95%.

$$U(C_{\alpha HCH}) = 2\ u_C\ (c_{capsaicin}) = 0.4260\ (c_{capsaicin})$$

Where $c_{capsaicin}$ is the concentration of analyte such as capsaicin, expressed in µg/L.

The developed method was applied to real samples of capsaicin in different chilli powder samples with several internal quality controls to ensure that the measurement process is under statistical control. Each batch of samples was processed together with a reagent blank, composed of only solvent. The reagent blank was obtained by performing the whole process without a sample. The majority of recoveries were in the range 90-110%. Calibration curves were prepared daily and the determination coefficients must be higher than 0.98.

The developed method was validated in order to ensure the feasibility of the method for its application in routine capsaicin analysis. Parameters such as linearity, recovery, precision and confirmation parameters such as MDLs, LOQs and uncertainty were studied.

Analysis of capsaicinoids in real samples

The proposed method was applied for the determination of capsaicin and dihydrocapsaicin in varieties of hot chili samples. The results of capsaicinoids analysis in some real chili samples are summarized in Table 1. Identification of compounds was achieved by retention time and absorption spectrum of standard and sample at 225 nm. The concentration of capsaicin as capsaicinoids was calculated from calibration curves.

Wilbur Scoville developed as scale in 1912 to measure the heat levels of the chili peppers. In the original, Scoville test (Scoville, 1912), a panel of volunteers would be asked to determine what dilution of chili pepper solution no longer cause burning discomfort in the mouth. Approximately one part per million of “heat” is equivalent to 1.5 Scoville units. The total capsaicinoids have been calculated on percent dry weight basis. For conversion of present capsaicinoids to SHU results been multiplied with 1,50,000 (i.e.1.5 lakh).

It is observed that that concentration of capsaicinoids determined in real chilli samples collected from the market ranged between 0.18 to 0.82%. Samples 1,2,3,4, and 15 showed higher concentrations .These varieties were typical and were obtained from nearby chilli cultivation egion such as Bhiwapur. The pungency of these samples were also determined and expressed in SHU. The results are summarized in Table1. The pungency ranged between 25500 and 124290 respectively. Higher pungency indicates hot chilliness of the sample.

Conclusion

The developed method was applied to real samples of chili powder obtained from local market. Each batch of samples was processed together with a reagent blank, composed of only solvent. The reagent blank was obtained by performing the whole process without a sample. The majority of recoveries were in the range 90–110%. Calibration curves were prepared daily and the coefficient of correlation was higher than 0.98. The developed method was validated in order to ensure the feasibility of the method.. Parameters such as linearity, recovery, precision and confirmation parameters such as MDLs, LOQs and uncertainty were studied. The capsaicin content and the pungency of the samples expressed as SHU was also determined.

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