ISSN: 0067-2904

Online Two-Dimensional Liquid Chromatography for Determination of Selected Flavonoids in Wine Samples

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Received: 10/11/2022 Accepted: 1/5/2023 Published: 30/4/2024

Abstract

The development of two-dimensional chromatography systems is crucial in many scientific fields as it provides improved separation efficiency and selectivity of complex mixtures. By separating compounds based on two independent properties, these systems can offer a more detailed understanding of sample composition. This increased level of detail is particularly useful in industries such as pharmaceuticals, environmental analysis, and proteomics, where complex mixtures with similar properties need to be differentiated. A new chromatography system with two dimensions was developed to separate and quantify specific flavonoids in German wine samples. The first dimension used a homemade hydrophilic interaction liquid chromatographic column, while the second dimension used a reversed-phase liquid chromatographic column. This 2D ZIC-HILIC x RP technique was able to address the problem of peak overlapping that commonly occurs in one-dimensional liquid chromatography, especially for natural products. The study presents analytical and statistical data for both techniques, and suggests that the use of two-dimensional chromatography can improve the resolution of conventional one-dimensional liquid chromatography. In a conventional ZIC-HILIC-HPLC system, apigenin and naringenin were detected together but were successfully separated and measured using a 2DLC system. The limits of detection (LOD) for apigenin and naringenin were found to be 0.0705 µg/mL and 0.1300 µg/mL, respectively, while the limits of quantification (LOQ) were 0.2136 µg/mL and 0.3939 µg/mL, respectively. The recovery rate for both compounds was over 99%, with a relative standard deviation (RSD) of less than 1.5%. The linearity range for both compounds was between 0.005 µg/mL to 10 µg/mL, and the correlation coefficient (r2) was determined to be 0.9998. For hesperidin and rutin, the LOD and LOQ were found to be 0.0336 µg/mL and 0.1018 µg/mL, and 0.0120 µg/mL and 0.0363 µg/mL, respectively. The linearity range for both compounds was between 0.005 µg/mL to 12 µg/mL, and the correlation coefficient (r2) was determined to be 0.9999.

Keywords: Two-dimension liquid chromatography, hydrophilic interaction liquid chromatography, flavonoids, win, online HILIC x RP 2DLC System, ZIC-HILIC, apigenin, naringenin

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كروماتوغرافيا السائل ذات البعدين المستمر لتقدير بعض الفلافونويدات في عينات النبيذ

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قسم الكيمياء ، كلية العلوم ، جامعة بغداد

الخلاصة:

يعد تطوير نظم كروماتوغرافيا السائل ثنائية الابعاد أمرًا بالغ الأهمية في العديد من المجالات العلمية حيث توفر كفاءة فصل محسنة وانتقائية أفضل للنماذج المعقدة. من خلال فصل المركبات بناءً على خواص تحليلية مستقلة لكل بعد من البعدين ، يمكن أن تقدم هذه الأنظمة فهمًا أكثر تفصيلاً لتكوين العينة. هذا المستوى المتزايد من التفاصيل مفيد بشكل خاص في صناعات مثل المستحضرات الصيدلانية والتحليل البيئي ودراسة البروتينات ، حيث تحتاج الامزجة المعقدة بخصائص مشابهة إلى التمييز. تم تطوير نظام كروماتوغرافيا جديد ثنائية الابعاد لفصل وتقدير مركبات الفلافونويدات في عينات النبيذ الألمانية. استخدم في البعد الأول عمود كروماتوغرافيا للتفاعل المحب للماء محلي الصنع ، بينما استخدم في البعد الثاني عمود كروماتوغرافيا ذي طور معكوس. كانت تقنية (2D ZIC–HILIC x RP) هذه قادرة على معالجة مشكلة تداخل القمم الذي يحدث عادة في الكروماتوغرافيا السائلة أحادية البعد ، خاصة للمنتجات الطبيعية. تقدم الدراسة بيانات تحليلية وإحصائية لكلتا الطريقتين ، وتقترح استخدام النظام ثنائي الأبعاد يمكن أن يحسن دقة الكروماتوغرافيا السائلة التقليدية أحادية البعد. ، لم يتم فصل الابيجينين و النارنجينين في نظام ZIC–HILIC– HPLC التقليدي ولكن تم فصلهما وتقديرهما بنجاح باستخدام نظام 2DLC. تم العثور على حدود الكشف (LOD) للأبيجينين والنارنجينين 0.0705 ميكروغرام / مل و 0.1300 ميكروغرام / مل ، على التوالي ، بينما حدود التقديرالكمي (LOQ) كانت 0.2136 ميكروغرام / مل و 0.3939 ميكروغرام / مل ، على التوالي. نسبة الاسترداد لكلا المركبين أكثر من 99٪ ، مع انحراف معياري نسبي (RSD) أقل من 1.5٪. تراوح مدى الخطية لكلا المركبين بين 0.005 ميكروغرام / مل إلى 10 ميكروغرام / مل ، ومعامل االخطية (r^2) مقداره 0.9998. بالنسبة للهسبيريدين والروتين ، وجد أن LOD و LOQ يساوي 0.0336 ميكروغرام / مل و 0.1018 ميكروغرام / مل و 0.0120 ميكروغرام / مل و 0.0363 ميكروغرام / مل على التوالي. تراوح مدى الخطية لكلا المركبين بين 0.005 ميكروغرام / مل إلى 12 ميكروغرام / مل ، ومعامل الخطية (r^2) مقداره 0.9999.

1. Introduction

Sophisticated analytical techniques and intricate software are essential for advanced analytical chemistry due to the highly challenging nature of the samples being analyzed, such as food, industrial, and natural samples, with complex physiochemical characteristics[1]. Although chromatographers have utilized various methods, including hyphenated approaches, to address the complexity of the samples, the demands for more effective tools are increasing [2]. Two-dimensional liquid chromatography, which can improve separation and maintain repeatability and sensitivity, is a promising technology[3].

In online two-dimensional liquid chromatography, two retention-mechanism-based columns are linked using an automated switching valve with two sampling loops [4]. The first-dimension effluent is transferred to the second column through the loops, and the modulation time, which equals the analysis time of the second dimension, is the period between each valve flip [5, 6]. According to reports, the modulation time is between 2.2 to 4 times the standard deviation of the peak in the first dimension. To expedite the analysis process, the second column should be smaller than the first column, to prevent excessive aliquots from accumulating before each analysis [7, 8]. In this particular study, a ZIC-HILIC column was utilized in the first dimension (1DLC), and a C18 reversed-phase column was

used in the second dimension (2DLC) to achieve total separation of four flavonoid compounds, including two glycoside flavonoids (hesperidin and rutin) and two aglycone flavonoids (naringenin and apigenin), which have the following structures.

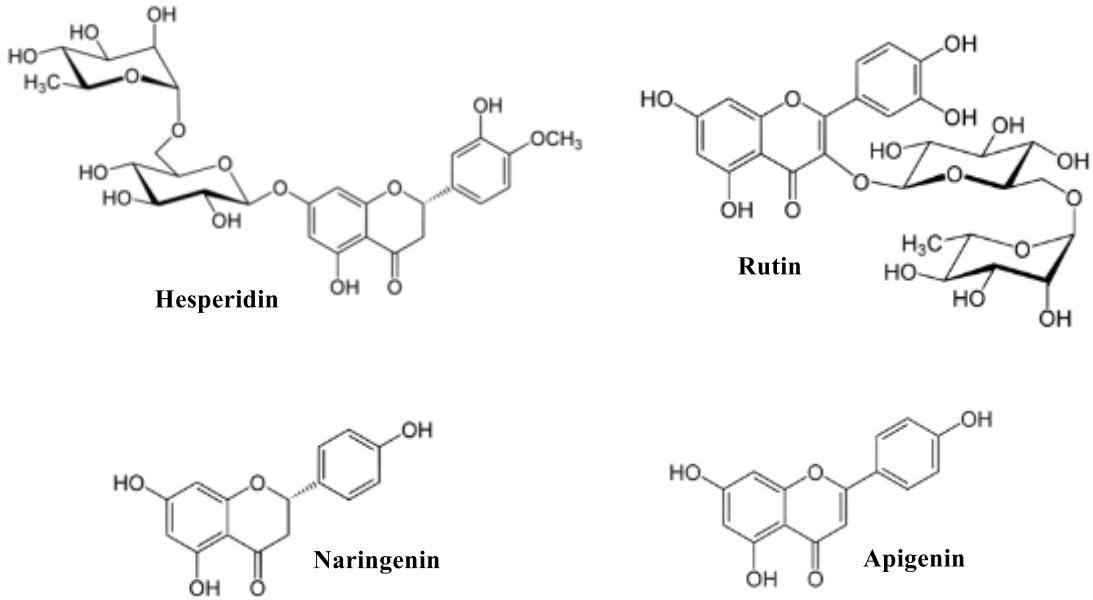

Figure 1: Structure of the selected flavonoids

Over the last 30 years, flavonoids have garnered significant attention and undergone thorough scientific investigations due to their natural biological properties, as well as their ability to scavenge free radicals and perform numerous bodily functions such as free radicals [9] and numerous bodily functions [10], including vasodilatory [11] anticarcinogenic [12], anti-inflammatory[13], antibacterial [14], innate immunity [15], antiallergic [16], antiviral [17], and hormone effects [18-20]. Wine, which is consumed globally, contains polyphenols such as phenolic acids, and their derivatives, and various flavonoids like flavones, flavanols, flavonols, and anthocyanins, making it an excellent subject for research [21, 22]. However, distinguishing and identifying distinct flavonoids in wine can be challenging. Reversed-phase liquid chromatography is the most commonly used technique for measuring wine polyphenols and is often combined with mass spectrometry, diode array detection, or tandem mass spectrometry. While mass spectrometry provides more sensitivity and structural information, RP-LC-DAD is preferred for selective detection [23, 24].

Sendtkowska compared various HILIC and RP modes and identified the optimal chromatographic conditions for four columns, including two HILIC (Atlantis-HILIC (100 mm × 2.1 mm) 3.0 μm and SeQuant ZIC-HILIC (100 mm × 2.1 mm) 3.5 μm) and two RP (fully porous (Luna C18 100 mm × 4.6 mm) 5 µm and core-shell (Kinetex C18 100 mm × 2.1 mm) 2.6 µm). Of the columns tested, SeQuant ZIC-HILIC showed the strongest retention of the analytes and was used to quantify flavonoids in Genista tinctoria Extract using an MS/MS detector [25, 26]. In another study, Sendtkowska investigated flavonoids in chamomile infusion using two different HILIC columns (Luna HILIC; 100 mm x 2.0 mm ID, 3.5 µm particle size, 200 Å and SeQuant ZIC-HILIC; 100 mm x 2.1 mm ID, 3 µm particle size, 135 Å). Under optimized conditions with ACN/Formic acid mobile phase and ammonium acetate buffer, the compounds hesperidin, hesperetin, rutin, quercetin, apigenin, luteolin, and genistein were successfully separated and detected, while naringenin was not included in the study [27].

Sendtkowska was able to isolate flavonoids from fruit juice using ZIC-pHILIC, a polymeric-based material. They tested various chromatographic conditions and mobile phases, including acetonitrile and methanol, with better sensitivity observed for naringenin using MeOH instead of acetonitrile [28]. Qiao employed an ionic liquid, N-diallyl-N-methyl-d-glucaminium bromide, to create a new stationary phase for column chemistry to separate flavonoids. This technique allowed for the separation and quantification of apigenin, naringenin, hesperidin, and others [29]. While HILIC is beneficial for flavonoid separation and quantification [3], combining HILIC and RP chromatography in a two-dimensional liquid chromatography (LC×LC) technique improves resolution, separation performance, and peak capacity, making it a powerful technique for analyzing complex phenolic fractions [30-33].

Duga et al. compared a traditional one-dimension HPLC method with a comprehensive two-dimensional liquid chromatography method using a C18 column to separate polyphenolic antioxidants in wine. The one-dimension chromatography utilized a 150 mm x 2.1 mm Discovery-HS column, while the two-dimension chromatography employed a micro-bore porous phenyl-silica column and a partially porous C18 column in the first and second dimensions, respectively (RPxRP-HPLC-DAD) [34]. The comprehensive 2DLC method resulted in a high degree of separation, with the ability to quantify multiple peaks co-eluted in a one-dimension chromatography. Jandera conducted a study on the characterization of HPLC columns in 2DLC for separating polyphenols, including flavonoids. The study examined six columns, none of which were HILIC columns, and was able to separate and quantify 33 compounds. The best 2DLC model used in the study consisted of a polyethene glycol microcolumn (PEG) as the first dimension and a short monolithic C18 column as the second dimension [35]. However, a drawback of 2DLC is that the first-dimension columns are often long and narrow, while the second-dimension columns are short, highly efficient, and have low flow resistance [36]. As an illustration, P. Jandera et al. synthesised monolithic microcolumns with very short columns (0.53 mm x 0.32 mm id) by copolymerizing an active monomer of zwitterionic sulfobetaine with the crosslinkers bisphenol A glycerolate dimethacrylate (BIGDMA) and dioxyethylene dimetacrylate (DiEDMA). In highly aqueous mobile phases and acetonitrile-rich mobile phases, the columns exhibit a dual retention mechanism (HILIC and RP modes) that can function in the first and second dimensions in 2DLC systems [37]. When tested for conventional 1DLC flavonoids analysis in HILIC and RP conditions, the columns themselves show some lack of ability to separate many flavonoids, including Apigenin and Naringenin in some cases. Still, when tested in the 2DLC technique by combining polar HILC columns in the first dimension and short RP columns in the second dimension, they met the 2DLC limitation requirements and showed excellent separation results.

2. Material and Methodology

2.1. Materials

Carl Roth Gmbh (Karlsruhe, Germany) provided the standards for hesperidin, rutin, apigenin, and naringenin, while Spelco supplied the HPLC grade solvents (including formic acid and acetonitrile (ACN)). Formic acid was used to adjust all mobile phases to a pH of 2.8 to prevent the ionization of phenolic functional groups. The mobile phase was filtered with 0.45 µm filters. The first-dimension column utilized a home-made ZIC-HILIC column, ZIC-SB5 (100 mm x 4.6 mm) 47 Å, particle size 3.5 µm, following the reference [38-40]. while the second-dimension column was obtained from Nouryon-Kromasil (Sweden), RP column, Kromasil C18 (50 mm x 3.0 mm id) 100 Å, particle size 5µm. The study was conducted with German red wine under the trade names (Anselmann pinot noir) and (Hensel Und Gretel) employing liquid-liquid extraction with an SPE cartridge.

2.2 Instrumentation

Utilizing an 844 UV/VIS compact I.C. instrument (Metrohm AG, Herisau, Switzerland), a one-dimensional liquid chromatographic analysis was conducted. Two 6-port switching valves were employed on the same instrument to execute comprehensive LC analyses. A 6-port 2-position switching valve model 708 sampling unit (Metrohm AG, Herisau, Switzerland) facilitated column switching. A schematic diagram depicting 2D HPLC (ZIC-HILIC x RP) is illustrated in Figure 5 with two 6-port switching valves. To link the ZIC-HILIC and RP columns, an electronically operated, two-position, and 6-port valve was used that encompassed two 40-L sample loops. The I.C. Net 2.3 software (Metrohm AG, Herisau, Switzerland) was utilized to regulate the switching valve and dimensions and consolidate and quantify the peaks.

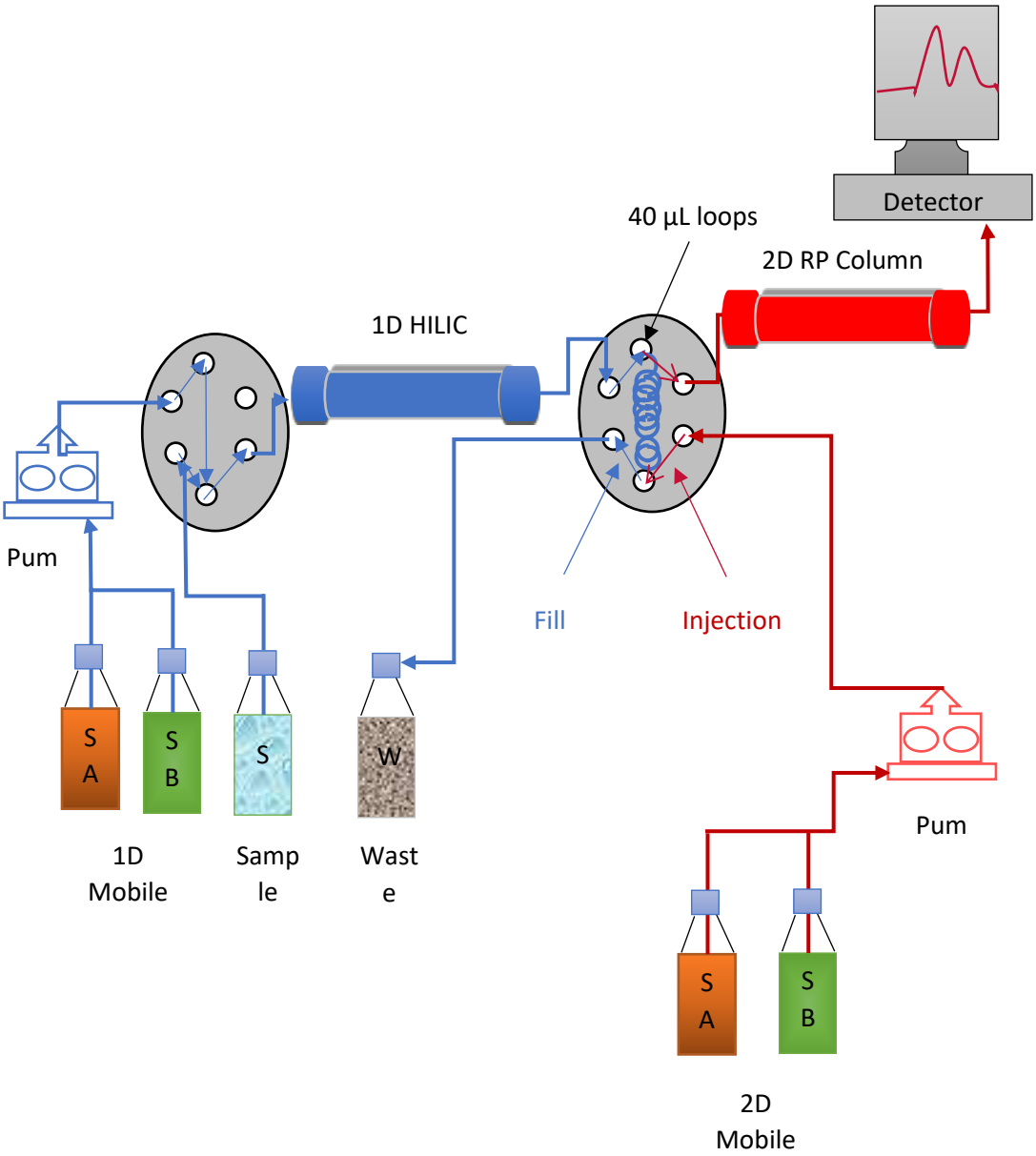

Figure 2: Schematic Diagram of 2D HPLC (ZIC-HILIC x RP) with Two 6-Port Switching Valve

2.3 Sample Preparation

The utilization of solid phase extraction employing a conditioned SPE cartridge confers several benefits, including rapidity, safety, and a high degree of selectivity. The initial preparation of the column involves introducing 6 mL of methanol, 2% formic acid, 5 mL of ACN, and 6 mL of water. Subsequently, the wine is loaded into the cartridge in increments of approximately 10 mL. Interfering materials are eliminated by applying 5 mL of ACN and 7 mL of water. 4 mL of ACN is subsequently added to the collected wine container. Evaluation of commercially available German-based grape wines (Anselmann pinot noir and Hensel Und Gretel), imported from Germany, was accomplished via direct analysis. Prior to injection, both samples were filtered through a 0.45 µm filter. The samples were suitably diluted with the corresponding mobile phases to allow for chromatographic analysis.

2.4 Method

2.4.1. One-dimensional Liquid Chromatography

A one-dimensional liquid chromatographic analysis was performed using a ZIC-SB5 column (100 mm x 4.6 mm; 3.5 µm). The mobile phase consisted of acetonitrile (A) and aqueous formic acid (B) (10 mM) under two different gradient conditions: condition (1)- 90%(A) and 10%(B) and condition (2)- 85%(A) and 15%(B). The analysis was conducted using a UV detector at a flow rate of 0.5 mL/min, and the resulting chromatograms were obtained at the wavelength of 350 nm.

2.4.2. Two-dimensional Liquid Chromatography

2DLC analyses were conducted utilizing a ZIC-SB5 column (100 mm x 4.6 mm ID) in the first dimension (ZIC-HILIC) and a C18 column (50 mm x 3.0 mm ID) in the second dimension (RP). The mobile phase consisted of two gradient conditions for the second dimension (RP), comprised of: A) acetonitrile and B) aqueous formic acid (10mM). These conditions were as follows: condition (3): 90%(A) and 10%(B), condition (4): 85%(A) and 15%(B). A 3.0 mL/min flow rate and a 35 C temperature were employed in D2. Chromatograms were retrieved at 350 nm. The 6-port valve was equipped with sample loops of 40 µL. The injection volume used in 1D and 2D LC studies was 10 µL.

2.4.3. Method validation

Calibration curves for hesperidin, apigenin, naringenin, and rutin were developed for use as reference materials for calculating the flavanone content of red wine (Figure 3). Ten concentration levels within the range of 0.005-12 µg/mL were generated by diluting a stock solution of 1000 µg/mL using acetonitrile as the solvent. The red wine samples were evaluated three times via 1D LC under identical chromatographic conditions. The reference materials for flavonoids were found to have excellent linearity, as shown in Table 1. For 2D LC analysis, eight to ten different concentrations in the 0.005-12 µg/mL range were generated for each of hesperidin, apigenin, naringenin, and rutin by diluting the same stock solution, and each concentration was examined in triplicate. The linearity of each combination in the 2DLC system is displayed in Table 1. The calibration curve's Limit of Detection (S/N = 3.3) and Limit of Quantification (S/N = 10) [41] were found to be within the concentration range of 0.012-0.13 µg/mL and 0.0363-0.3939 µg/mL, respectively, demonstrating excellent sensitivity. Furthermore, the system's linearity ranges from 0.005 µg/mL to 12 µg/mL, indicating a wide range of potential applications.

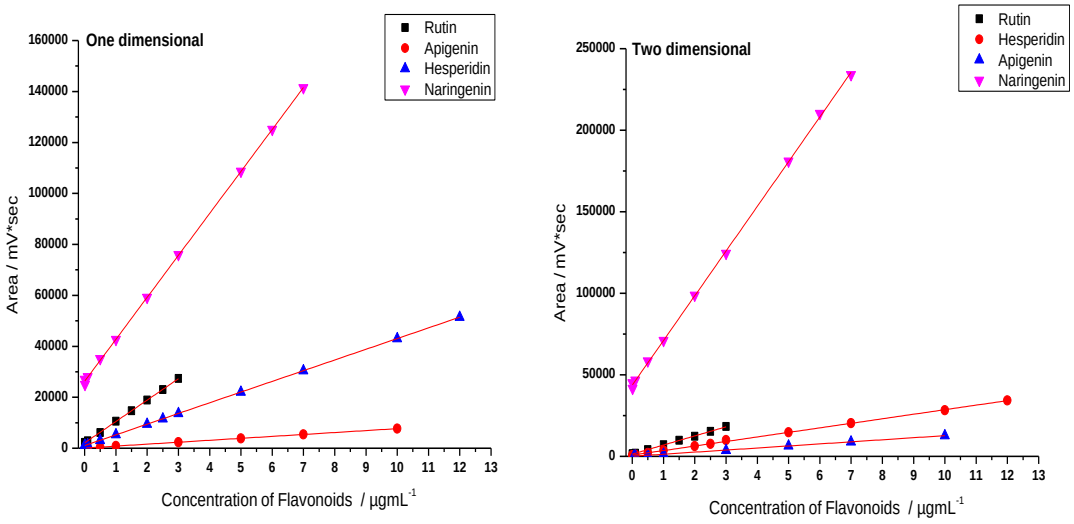

Figure 3: Calibration curves of 1DLC and 2DLC for rutin, hesperidin, apigenin, and naringenin

Table 1: Calibration data of 1DLC and 2DLC curves

Analyte	Method	Regression Equation	R^2	Linear range (µg/mL)	LOD (µg/mL)	LOQ (µg/mL)
Hesperidin	1D	y = 1047 + 4199 *x	0.9999	0.005-12	0.0024	0.0072
Hesperidin	2D	y = 701 + 2789 *x	0.9997	0.005-12	0.0336	0.1018
Apigenin	1D	y = 70.19 + 762 *x	0.9999	0.05-10	0.0047	0.0142
Apigenin	2D	y = 67 + 1262 *x	0.9995	0.05-10	0.0705	0.2136
Naringenin	1D	y = 26279 + 16472 *x	0.9998	0.05-7	0.0130	0.0390
Naringenin	2D	y = 43687 + 27408 *x	0.9996	0.05-7	0.0820	0.2484
Rutin	1D	y = 2095 + 8398 *x	0.9997	0.005-3	0.0010	0.0030
Rutin	2D	y = 1396 + 5598 *x	0.9995	0.005-3	0.0120	0.0363

2.4.4. Evaluation of the Developed Method

Precision and stability are defined as critical factors in determining the effectiveness of analytical performance [42]. Evaluations of analytical performance demonstrate that the proposed analytical methodology achieves satisfactory outcomes. This evaluation provides insights into the quality, reliability, and consistency of the analytical findings. To assess precision, the repeatability of the proposed methodology was evaluated by conducting analyses for three consecutive days. The RSD values for both intra- and inter-day analytical findings were computed and found to be less than 1.5%, indicating a remarkable level of repeatability. The precision of the method was quantified by expressing RSD values in relation to the percentage recovery. The results presented in Table 2 confirm the accuracy of the proposed analytical methodology.

Table 2: Statistical data for method evaluation

Analyte	Taken µg/mL	ID				2D			
		Inter-Day		Intera-Day		Inter-Day		Intera-Day	
		Rec. (%)	RSD (%)	Rec. (%)	RSD (%)	Rec. (%)	RSD (%)	Rec. (%)	RSD (%)
Hesperidin	0.5	98.60	0.23	98.20	0.36	98.60	0.41	99.60	0.50
	1	99.10	0.36	99.40	0.45	99.40	0.53	99.60	0.71
	1.5	99.33	0.30	99.40	0.37	100.33	0.24	100.60	0.44
Apigenin	0.5	99.00	0.32	99.00	0.39	101.00	0.78	101.40	1.01
	1	98.80	0.44	99.10	0.54	99.70	1.06	99.70	1.26
	1.5	99.46	0.22	99.33	0.29	100.06	1.33	100.26	1.45
Naringenin	0.5	98.00	1.04	99.00	1.01	99.20	1.23	99.20	0.98
	1	96.00	0.55	99.30	0.65	98.50	0.33	99.10	0.75
	1.5	100.46	0.82	100.20	0.87	100.60	0.95	100.33	0.37
Rutin	0.5	97.80	0.48	98.00	0.56	98.80	0.51	99.20	0.87
	1	97.10	0.34	97.10	0.38	102.00	0.63	101.50	0.60
	1.5	98.46	0.28	98.86	0.31	99.33	0.34	99.33	0.52

Statistical analyses were performed with a confidence level of 95%, utilizing the outputs of the t-test method and variance ratio F-test method (Table 3). A comparison was made between these results and those obtained from the traditional method, in order to evaluate the efficacy and competence of the 2D method in relation to the conventional method [43]. The computed T and F values were found to be within the theoretical values, suggesting that the accuracy of flavonoid quantification does not vary significantly between the two methods.

Table 3: The comparison of the proposed methods 2D with traditional method's for flovonoides analysis by examining t- and F-statistical tests

Flovonoides	Taken µg/mL	2D	Traditional method	t-Test (theor.)	F-Test (theor.)
Hesperidin	0.5	98.60	99.22	0.7513 (2.7764)	3.3580 (19.000)
	1	99.40	100.15		
	1.5	100.33	99.54		
Apigenin	0.5	101.00	100.65	0.4975 (2.7764)	0.8970* (19.000)
	1	99.70	99.47		
	1.5	100.06	99.38		
Naringenin	0.5	99.20	100.44	0.8977 (2.7764)	1.1032 (19.000)
	1	98.50	99.77		
	1.5	100.60	98.44		
Rutin	0.5	98.80	99.33	0.7062 (2.7764)	2.6721 (19.000)
	1	102.00	101.33		
	1.5	99.33	100.88		

3. Results and Discussion

The present investigation endeavours to establish a qualitative and quantitative approach to segregate and evaluate specific flavonoids present in red wine. This objective was

accomplished utilizing HPLC-ZIC-HILIC x RP-UV technique in online comprehensive two-dimensional liquid chromatography.

3.1. Analysis of flavonoids by ZIC-HILIC 1DLC:

The retention time of four mixed reference materials flavonoids isolated via ZIC-SB5 column in one dimension mode is summarized in Table 4. The inefficiency of separating the naringenin and the apigenin flavonoids using conventional chromatography is evident due to similar hydroxyl groups in the same positions of both flavonoids. The ZIC-SB5 column facilitated flavonoid separation in wine samples in just 16 minutes and produced a good baseline under gradient chromatographic conditions as shown in Figure 1.

Table 4: Retention time of mixed flavonoids standards

Flavonoid	Retention time (t_R min)
Apigenin	3.32
Naringenin	3.38
Rutin	15.11
Hesperidin	9.72

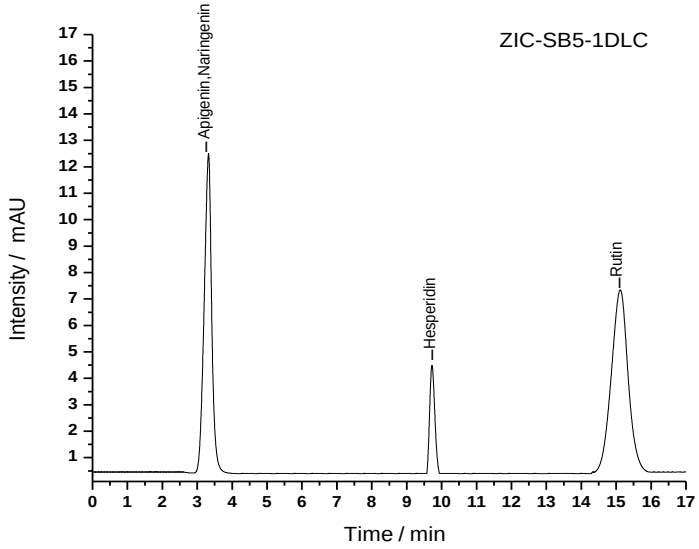

Figure 4: The chromatogram of the ZIC-HILIC column for separating the four flavonoids.

3.2. Analysis of flavonoids by ZIC-HILIC x RP 2DLC:

This study highlights the advantage of two-dimensional liquid chromatography in increasing the peak capacity and providing higher resolution, especially for those co-eluted in conventional liquid chromatography. ZIC-HILIC and RP mechanism exhibited high orthogonality guaranteeing the success of two-dimensional liquid chromatographic separation. ZIC-SB5 was used in the 1D analysis, while RP C18 in the 2D, providing higher peak capacity and better resolution for apigenin and naringenin, as evidenced by retention time and chromatogram in Table 5 and Figure 2, respectively.

Table 5: Retention time of mix flavonoids standards by ZIC-HILIC x RP 2DLC

Flavonoid	Retention time (t_R min)
Apigenin	23.63
Naringenin	20.19
Hesperidin	15.71
Rutin	12.36

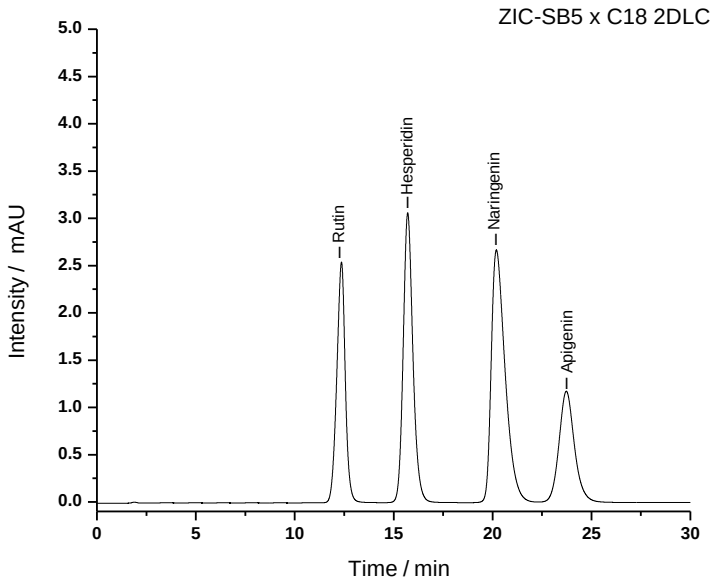

Figure 5: The chromatogram of ZIC-HILIC x C18 2DLC for separating the four flavonoids.

3.3 Determination of flavonoids in wine samples

Hesperidin, rutin, apigenin, and naringenin concentrations in two wine samples were successfully quantified using the suggested two-dimensional liquid chromatography technique (Table 6). ZIC-HILIC x RP 2DLC separation was demonstrated to investigate bioactive components in extracts of two wine samples (Anselmann pinot noir-Germany and Hensel Und Gretel-Germany under chromatographic conditions 90% A and 10%B for the first dimension and 20% and 80%B for the second dimension.

Table 6: The flavonoid contents in red wine

Flavonoids				
Wine samples	**Apigenin mg/100 mL[a]**	**Rutin mg/100 mL[a]**	**Naringenin mg/100 mL[a]**	**Hesperidin mg/100 mL[a]**
1- Anselmann pinot noir	0.220 ± 0.530	1.105 ± 0.050	2.500 ± 0.230	0.019 ± 0.650
2- Hensel Und Gretel	0.580 ± 0.732	0.320 ± 0.108	1.788 ± 0.440	0.054 ± 0.180

a.Contents (mg/100 mL) as mean + SD, are expresses (n = 3).

Conclusion

Flavonoid separation and quantification in various samples are commonly achieved through the use of reversed-phase liquid chromatography (1DLC-RP) and hydrophilic interaction liquid chromatography (1DLC-HILIC). However, the identification of flavonoids with similar polarity in complex samples often results in peak overlap when employing traditional 1DLC-RP or 1DLC-HILIC. To address this limitation, a new two-dimensional liquid chromatography (2DLC) system has been developed utilizing a first dimension ZIC-HILIC column and a second dimension RP-C18 column. This system provides excellent separation abilities for the analysis of flavonoids in wine samples, allowing for the resolution of overlapped peaks of similarly polar compounds by transferring fractions of already separated aliquots from the 1D column to the 2D column via a controlled switching valve. This enhances the separation efficiency and facilitates orthogonal separation mechanisms for analytes. Flavonoids in wine samples were analyzed using both the conventional 1D ZIC-HILIC and the 2D ZIC-HILIC x RP. Among the tested flavonoids, apigenin and naringenin

were found to co-elute using the traditional method but were successfully separated and quantified in microgram levels with the new 2DLC system. Method evaluation demonstrated good sensitivity and reliable repeatability, indicating that the newly designed 2DLC method is precise and applicable, with no statistically significant differences when compared to traditional methods.

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