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Simultaneous quantification of ferrous gluconate and calcium gluconate in foods using liquid chromatography–tandem mass spectrometry (LC–MS/MS)

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Abstract

Ferrous gluconate and calcium gluconate are used as food acidity regulators in South Korea, Japan, the European Union (EU), and other countries. A simultaneous analytical method was developed to quantify ferrous gluconate and calcium gluconate in food using ultra-performance liquid chromatography–tandem mass spectrometry. The limits of detection and quantification of ferrous gluconate were 1.1 and 3.5 mg/kg, respectively, while those of calcium gluconate were 1.4 and 4.8 mg/kg, respectively. The recoveries of ferrous gluconate from processed olives were in the range of 97.7–109.7%, while those of calcium gluconate from beverages were in the range of 94.3–110.8%. The developed simultaneous analytical method was applied to real samples from South Korea, which found ferrous gluconate concentrations of 0.031–0.065 g/kg in processed olives and calcium gluconate concentrations of 3.8–7.8 g/kg in beverages.

Keywords Ferrous gluconate, Calcium gluconate, Food acidity regulator, Liquid chromatography–tandem mass spectrometry, Simultaneous analysis

Introduction

Acidity regulators are food additives used to change or maintain the pH of food to prevent discoloration or oxidation occurring during storage [1]. Ferrous gluconate (FG) and calcium gluconate (CG) are widely allowed food acidity regulators, including in South Korea, Japan, the United States, the European Union (EU), and Codex member countries [1–5]. FG and CG are water-soluble

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compounds in which two gluconate molecules are bound to iron or calcium centers, respectively (Fig. 1). FG is a yellowish gray to greenish-yellow solid with a slightly peculiar odor, while CG is a white crystalline or granular solid that is odorless and tasteless [1]. The median lethal doses (LD_{50}) of FG for oral administration in rats and mice are 2.2 and 3.7 g/kg of body weight, respectively [6, 7], while the LD_{50} of CG for oral administration in rats is 5.0 g/kg of body weight [8, 9]. The provisional maximum tolerable daily intake (PMTDI) of FG established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) is 0.8 mg/kg of body weight, while none has been established for CG [2].

FG is an acceptable food acidity regulator for processed olives in South Korea, Codex member countries, the EU, and Japan, with a usage level set at an iron content of 0.15 g/kg or below, while its use in Canada is set at the Good Manufacturing Practice (GMP) level [1–3,

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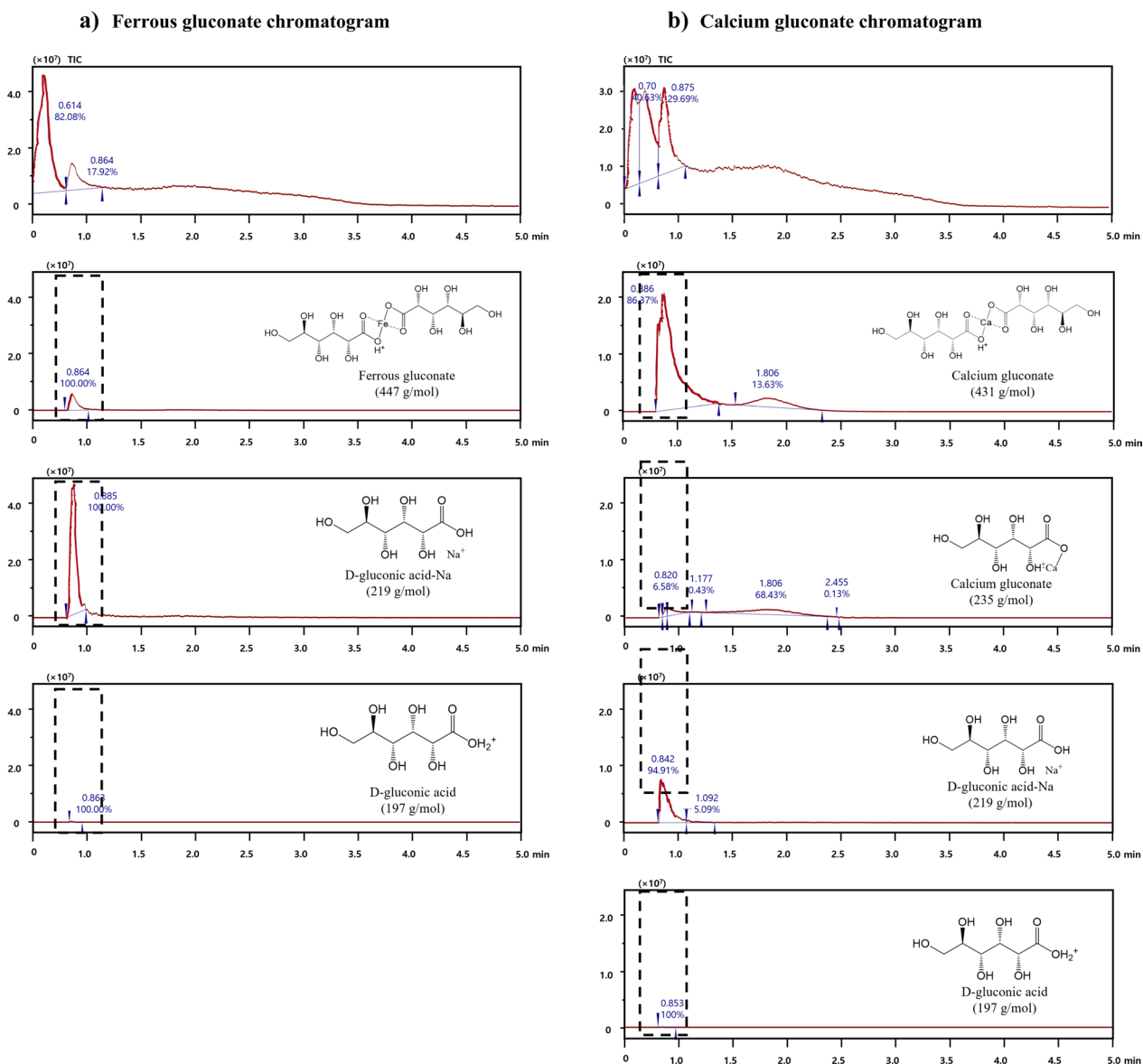
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Fig. 1 Chromatograms of FG and CG in positive mode using UPLC–MS

5, 10]. CG can be used in all foods in South Korea and Japan, with its usage level set at a calcium content of 10 g/kg or below [1, 3]. In Codex member countries, the EU, and Canada, CG use at the GMP level is allowed in processed cheese, dairy products, and canned fruits and vegetables [5, 10, 11]. Although the use of FG and CG is acceptable in foods, adequate and quantitative methods for FG and CG contained in food have yet to be developed. Therefore, regulations for the use of FC and CG in food have been established based on iron and calcium levels instead of FG and CG levels.

Yu-li [12] reported a quantitative method for FG contained in pharmaceuticals using HPLC with an ultraviolet–visible detector (UVD) and reversed-phase column (Zorbax SB C_{18}). Nikolić et al. [13] also performed quantitative analysis of FG using HPLC with a UVD at 205 nm and C_{18} column (Zorbax Eclipse XDB). Meanwhile, Mahgoub [14] analyzed the CG content in pharmaceuticals at 210 nm using HPLC with a C_{18} column (Inertsil ODS) and UVD. Previous studies have not included matrix interference effects on FG or CG, and no validation of the analytical methods has been reported, which limits its application to food matrices. As shown in Fig. 1,

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owing to FG and CG having very similar structures and solubility characteristics in solvents, separating their peaks might be difficult when both materials are used together. Accurately identifying FG and CG molecules in the food matrix and quantifying their individual contents is considered the optimal technique for quantification of these two additives. However, the simultaneous analysis of FG and CG derivatives using liquid chromatography–tandem mass spectrometry (LC–MS/MS) has not been reported to date.

Therefore, in this study, a method for analyzing FG and CG simultaneously using ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) was developed, which allowed identification and quantification of the main derivatives of the two additives for the first time. Furthermore, the validity of the analytical methods was verified by assessing the limit of detection (LOD), limit of quantification (LOQ), linearity, matrix effects, accuracy, precision, and sample monitoring.

Materials and methods
Reagents
Ferrous gluconate standard (99.0% purity) and calcium gluconate standard (98.0% purity) were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade water, methanol, and acetonitrile were obtained from Sigma-Aldrich. Trifluoroacetic acid and a 0.2-µm polyvinylidene difluoride (PVDF) filter were purchased from Alfa Aesar (Boston, USA) and Whatman (Maidstone, England), respectively.

Sample preparation
The method for extracting FG and CG from samples was optimized in this study. To extract FG and CG from food, samples (processed olive products and beverages) that did not contain FG and CG were purchased and a recovery test was conducted. A processed olive sample (blank, 1 g) not containing FG was placed into a tube and spiked with the FG standard to 1000 µg/mL. Furthermore, a beverage (blank, 1 g) not containing CG was precisely weighed into a tube and spiked with CG standard to 1000 µg/mL. Water (40 mL) was added to the olive products and beverage samples fortified with FG and CG, respectively, followed by extraction for 10 min using an ultrasonicator (UC-20, Lab Companion, Billercia). After centrifugation at 10,000 rpm for 10 min (VS-550, Vision Scientific Co., Daejeon, South Korea), the upper layer was collected and filtered through a 0.2-µm PVDF filter (Maidstone, England). The filtrate was analyzed by UPLC-MS/MS. In the present study, instead of water, 1.0% trifluoroacetic acid, and a mixture of 1.0% trifluoroacetic acid and acetonitrile (95:5, v/v), were added for

extraction and the recovery rates were compared. When water was used as the extraction solvent, the recovery rates of FG and CG were 101.6% and 95.9%, respectively. Using 1.0% trifluoroacetic acid, and a mixture of 1.0% trifluoroacetic acid and acetonitrile (95:5, v/v), the recovery rates of FG were 94.8% and 96.0%, respectively, while those of CG were 28.0% and 93.2%, respectively. Therefore, water was the solvent with the highest extraction efficiency. Subsequently, all samples were extracted with water and stored at -4 °C prior to analysis, which was performed within 1–2 days.

UPLC-MS/MS analysis
Identification and quantitative analysis of FG and CG were performed using a UPLC-MS/MS system (Waters Xevo TQ-S micro, Milford, MA, USA) with electrospray ionization (ESI) as the ionization source. Chromatographic separation was performed using a UPLC system (Waters Synapt G2-S, Acquity UPLC, Waters Corporation, Milford, MA, USA) with an Acquity UPLC BEH C18 column (1.7 µm, 100 mm × 2.1 mm, Waters Corporation, Milford, MA, USA). The mobile phase consisting of water (A) and acetonitrile (B) each containing 0.1% trifluoroacetic acid were prepared under gradient conditions (0–4 min, 20% A/80% B; 4–5 min, 100% B), and the flow rate was 0.3 mL/min.

LC–ESI–MS/MS analysis was performed by selecting the parent ion as $[M+H]^+$ in positive-ion ESI mode, and data were collected using Mass Lynx v4.1 (Waters, Manchester, UK). Detailed ESI–MS/MS conditions were as follows: spray voltage, 3.0 kV; cone voltage, 28 V; source gas flow rate, 650 L/h; source temperature, 150 °C; desolvation gas temperature 350 °C. Collision-induced dissociation was carried out using argon gas. The multiple reaction monitoring (MRM) transitions and collision energy (CE) for FG and CG were shown in Table 1.

Method validation
To verify the simultaneous analytical method for FG and CG developed in this study, six validation parameters, namely, linearity, LOD, LOQ, accuracy, precision, and recovery rate, were checked based on the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use [15–17] and Korea MFDS guidelines [18]. For method validation, processed olive products and noncarbonated beverages were purchased from a local market in Cheonan, South Korea. Standard solutions of FG and CG were prepared at concentrations between 50 and 500 µg/mL, and analyzed by UPLC–MS/MS to obtain calibration curves (C1). Spiked samples were prepared by spiking blank samples that did not contain FG and CG with the same concentrations (between 50 and 500 µg/mL of FG and

Table 1 MRM transition and optimized parameters of LC–MS/MS for FG and CG

Compound	Ion mode (ESI+/−)	Molecular weight(g/mol)	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (eV)	Cone voltage (V)	Retention time (min)
Ferrous gluconate	+	446.1	447	411[a]	22	28	0.79
				293	14	28	0.79
				205	36	28	0.79
				191	12	28	0.79
				175	14	28	0.79
Calcium gluconate	+	430.4	431	333[a]	24	28	0.73
				317	15	28	0.73
				235	28	28	0.73
				175	34	28	0.73
				159	34	28	0.73

[a] Quantification ion

CG). To confirm matrix effects, spiked samples were analyzed by UPLC–MS/MS to prepare further calibration curves (C2). Each sample was injected in three replicates. The linearities of C1 and C2 were investigated by measuring the correlation between concentrations obtained by LC–MS/MS analysis.

The LOD and LOQ were obtained from the standard deviation of the response and the slope of the calibration curve of the standard mixture at low concentrations (1, 5, and 10 μg/mL), as described in the ICH guidelines [17]; LOD (limit of detection) = 3.3 σ/S and LOQ (limit of quantification) = 10 σ/S, where σ is the standard deviation and S is the slope of the standard curve.

Accuracy and precision were obtained from spiked samples at concentrations between 50 and 400 μg/mL through intra- and inter-day analysis. To assess intra-day accuracy and precision, known amounts of FG were spiked into the processed olive products at final concentrations of 50, 100, 200, 300, and 400 μg/mL, while CG was spiked into the noncarbonated beverages at these same five concentration levels. All prepared samples were analyzed five times within the same day. The mean concentrations analyzed from the spiked samples were compared with the theoretical concentration, and the recoveries were calculated as the accuracy. Relative standard deviations (RSD) were calculated as the precision, which represents repeatability. For inter-day accuracy and intermediate precision, known amounts of FG were spiked into the processed olive products at final concentrations of 50, 100, 200, 300, and 400 μg/mL for 3 days, while CG was spiked into the noncarbonated beverages at these same five concentration levels. The recoveries from all spiked samples (processed olive products and noncarbonated beverages) were analyzed for 3 separate days. The intermediate precision

was determined using the relative standard deviation obtained by inter-day analysis.

Cross-laboratory tests were conducted on spiked samples of 50, 200, 300, 400, and 500 μg/mL for FG and CG at two different laboratories. Linearity, accuracy (recovery), and precision (RSD) were obtained by triplicate measurement of each sample. All spiked samples were analyzed in two different laboratories using the same pretreatment method and simultaneous LC–MS/MS conditions developed in this study. The calibration curve linearity, accuracy obtained at each laboratory, and RSD (%) values between laboratories were calculated according to the ICH guidelines [17].

Application of the method to real samples

Five types of processed olive product containing FG and five types of noncarbonated beverage containing CG were purchased from local markets in Cheonan, South Korea. The contents of FG and CG in the purchased samples were determined by applying the pretreatment method and LC–MS/MS conditions developed in this study.

Results and discussion
LC–MS/MS method development

As a preliminary step to optimize FG and CG analysis, FG in processed olives and CG in beverages were analyzed according to the HPLC method reported in previous studies [12, 13] by additionally changing column types such as C18, C4, and ODS columns using different mobile phase, and gradient conditions. In all experiments, the FG and CG peaks were detected as one peak without peak separation and the peaks of FG and CG overlapped exactly with gluconate (data not shown). In the case of CG, peak interference was also observed by the matrix of beverage. Therefore,

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the LC–MS/MS analysis method was finally selected because it can analyze single molecules in MRM mode without perfect peak separations in TIC mode. Simultaneous analysis of FG and CG could be archived by UPLC–MS/MS (Waters Synapt G2-S, Acquity UPLC) with Acquity UPLC BEH C18 columns. The mobile phase giving the best resolution was a gradient system using water (A) and acetonitrile (B) containing 0.1% trifluoroacetic acid, respectively (0–4 min, 20% A/80% B; 4–5 min, 100% B). The mobile phase consisting high concentration of acetonitrile and water could lead the CG or FG in bound form with calcium or iron, respectively, to make $[M+H]^+$ precursor ions in ESI. To obtain MS/MS spectra, standard solutions of FG and CG (100 mg/mL) were separately injected into the ESI source by continuous injection in positive ion mode. In the total ion chromatogram (TIC), peaks for FG and CG were found between 0.8 and1.4 min without complete peak separations, as shown in Fig. 1. Therefore, FG and CG were further analyzed in multiple reaction monitoring (MRM) mode. All parameters have been optimized to maximize sensitivity and reproducibility of target analytes. The spray voltage (3.0 kV) and capillary temperature (350 °C) were chosen so that all compounds exhibit good mass response. When the collision energy was increased to 55.0 eV, all precursor ions were separated into unstable fragment ions, which were not suitable for quantitative analysis. Therefore, careful attention was paid to the collision energy settings, and the multiple reaction monitoring (MRM) transitions and collision energies (CE) of the analytes were obtained as presented in Table 1.

The most abundant product ions in terms of better sensitivity for FG and CG were found to be m/z of 411 and 333, respectively, to obtain the highest signal intensity for the analytes (Table 1). The recovery ratios of FG and CG in food matrices using selected MRMs were over 97.7% and 94.3%, respectively (Table 3).

Method validation

To verify the simultaneous LC–MS/MS method for quantitative analysis of FG and CG, the linearities of the calibration curves (C1) of each chemical were determined using the standard solutions of FG and CG with concentrations of 100, 200, 300, 400, and 500 μg/mL. Both calibration curves of FG and CG standards showed excellent linearity ($R2 > 0.99$). The LODs of FG and CG were 1.1 and 1.4 μg/mL, while the LOQs were 3.5 and 4.8 μg/mL, respectively (Table 2). Herein, the LODs and LOQs were calculated using statistical methods [8, 18].

Other calibration curves (C2) were determined by analyzing blank samples fortified with 100, 200, 300, 400, and 500 μg/mL of FG and CG standards. These two matrix-matched calibration curves obtained from fortified samples also showed excellent linearity ($R^2 > 0.99$). The method detection limits (MDLs) of FG and CG were 3.7 and 8.6 μg/mL, while the method quantification limits (MQLs) were 12.2 and 26.0 μg/mL, respectively (Table 2). The MDLs and MQLs were calculated using statistical methods [8, 18].

To evaluate matrix effects, the slope of the calibration curve obtained from the standard solution (C1) was compared with that of the matrix-matched calibration curve obtained from spiked samples (C2; prepared by applying the method described in a previous study) [19]. For both FG and CG, a significant difference was observed between the slopes of the standard calibration curve and matrix-matched calibration curve. To confirm the differences between these two calibration curves, the variance and averages of the two calibration curves were compared using the F-test and t-test, with the results shown in Table 2. As a result of the F test between the two calibration curves for FG, a significant difference was observed in the variance of the two slopes, and the same result was found for CG. These results showed that there was a matrix effect on the linear response of the calibration curves in FG and CG analysis. Therefore,

Table 2 Results for F and t-tests of the calibration curves for FG and CG

	Ferrous gluconate	Calcium gluconate	Tabled value
R^2 (C1)	0.997 (y = 729.67x − 39978)	0.994 (y = 1609.7x − 99182)	–
LOD	1.1	1.4	–
LOQ	3.5	4.8	–
R^2 (C2)	0.994 (y = 14.684x + 2431.9)	0.996 (y = 9.97x + − 1024.3)	–
MDL	3.7	8.6	–
MQL	12.2	26.0	–
t-test	− 3.1068 (p < 0.027)	− 3.0995 (p < 0.027)	3.1825
F-test	0.0004 (p < 0.00001)	0.00003 (p < 0.0000004)	0.1078

R^2: correlation coefficients; C1: calibration curve for standard solution; C2: matrix-matched calibration curve; MDL: method detection level; MQL: method quantification level

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Table 3 Results of accuracy and precision for FG and CG using simultaneous LC–MS/MS method

Conc. (µg/mL)	Ferrous gluconate					
	Intra-day test (n = 5)			Inter-day test (n = 3)		
	Measured concentration (µg/mL)	Accuracy (recovery%)	Precision (RSD%)	Measured concentration (µg/mL)	Accuracy (Recovery%)	Precision (RSD%)
50	54.3 ± 0.8	108.6	1.5	54.9 ± 1.3	109.7	2.4
100	104.4 ± 0.4	104.4	0.4	107.8 ± 0.4	107.8	0.3
200	207.8 ± 4.3	103.9	2.1	204.2 ± 0.9	102.1	0.4
300	304.0 ± 4.9	101.3	1.6	294.8 ± 2.0	98.3	0.7
400	390.8 ± 8.7	97.7	2.2	409.6 ± 5.5	102.4	1.3
Conc. (µg/mL)	Calcium gluconate					
	Intra-day test (n = 5)			Inter-day test (n = 3)		
	Measured concentration (µg/mL)	Accuracy (recovery%)	Precision (RSD%)	Measured concentration (µg/mL)	Accuracy (recovery%)	Precision (RSD%)
50	52.5 ± 3.4	105.0	6.4	55.4 ± 1.0	110.8	1.8
100	100.1 ± 3.0	100.1	3.0	94.3 ± 1.3	94.3	1.4
200	206.4 ± 4.1	103.2	2.0	202.4 ± 1.5	101.2	0.8
300	303.0 ± 3.3	101.0	1.1	311.4 ± 4.3	103.8	1.4
400	393.2 ± 6.2	98.3	1.6	403.1 ± 4.5	100.8	1.1

all subsequent validation parameters for LC–MS/MS analysis of FG and CG were analyzed using the matrix-matched calibration curves.

In the intra- and inter-day tests, the accuracies were evaluated using recovery tests of olives spiked with FG (or noncarbonated beverages spiked with CG) at final concentrations of 50, 100, 200, 300, and 400 µg/mL. The recoveries of FG from olives ranged from 97.7 to 109.7% in the intra- and inter-day tests. The recoveries of CG from noncarbonated beverages in the intra- and inter-day tests were in the range of 94.3–110.8% (Table 3). These FG and CG recovery rates satisfied the criterion of 70.0–120.0% recommended by the EU directive [20].

Here, RSD in intra- and inter-day tests were calculated as the repeatability or precision. Repeatability was evaluated using the RSD obtained from intra-day recovery experiments for FG and CG at five concentrations, affording results of 0.4–2.2% and 1.1–6.4%, respectively. Intermediate precision was evaluated using the RSD obtained from inter-day recovery tests for FG and CG (0.3–2.4% and 0.8–1.8%, respectively), which were lower than the value of 20% recommended by the EU directive [20].

To validate the simultaneous LC–MS/MS method for FG and CG determination, cross-laboratory tests were performed on samples spiked at five concentrations at two laboratories, which followed the criteria recommended by the Joint FAO/IAEA Expert Consultation [21]. Herein, the spiked samples were prepared by spiking 50, 200, 300, 400, and 500 µg/mL of FG standard solution into blank olives (not containing FG) and CG standard solutions (50, 200, 300, 400, and 500 µg/mL) into blank beverages (not containing CG), respectively. The cross-laboratory recoveries of FG and CG from foods using the simultaneous LC–MS/MS method are shown in Table 4. For FG, the recovery ranged from 95.2 to 109.7%, with the RSD ranging from 0.7 to 9.0%, for the five concentrations in the olive matrix. For CG, the recovery ranged from 97.4 to 103.7%, with the RSD ranging from 0.9 to 1.7%, for the five concentrations in the beverage matrix. These results satisfied the accuracy criterion of 70–120% set by the Codex Alimentarius Commission, while the RSD results met the 20% value recommended by the EU directive [20, 22].

The developed simultaneous LC–MS/MS method was used to determine FG and CG in processed olive products and beverages purchased from local Korean markets. The results for FG and CG obtained from individual sample analyses are shown in Table 5. From the analysis of both FG and CG in ten samples each, FG was detected in all olive products originating from Italy and Spain, while CG was detected in all Korean beverages samples. The FG and CG concentrations of the positive samples were 0.031–0.065 g/kg and 3.8–7.8 g/kg, respectively,

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Table 4 Results of recoveries of FG and CG in foods using simultaneous LC–MS/MS method

	Lab 1		Lab 2		RSD (%)
	Measured concentration (µg/mL)	Recoveries (%)	Measured concentration (µg/mL)	Recoveries (%)	
Ferrous gluconate					
Concentration (µg/mL)					
50	54.9 ± 1.3	109.7	48.3 ± 3.5	96.6	9.0
200	205.3 ± 5.7	102.6	202.1 ± 5.9	101.0	1.1
300	296.0 ± 2.1	98.7	302.7 ± 5.2	100.9	1.6
400	405.4 ± 2.3	101.4	380.8 ± 9.8	95.2	4.4
500	501.9 ± 4.1	100.4	506.6 ± 3.8	101.3	0.7
R^2 (correlation coefficient)	0.997		0.997		
Calcium gluconate					
Concentration (µg/mL)					
50	51.1 ± 3.9	102.2	51.8 ± 2.9	103.7	1.0
200	202.9 ± 1.8	101.5	198.0 ± 0.9	99.0	1.7
300	301.3 ± 1.9	100.4	305.2 ± 0.5	101.7	0.9
400	395.0 ± 6.9	98.8	389.7 ± 8.1	97.4	1.0
500	498.8 ± 5.5	99.8	505.7 ± 2.4	101.1	1.0
R^2 (correlation coefficient)	0.998		0.997		

Table 5 Analysis results of FG and CG in processed olive and non-carbonated beverages

Sample information (originated location)	Contents of ferrous gluconate (g/kg)	Contents of calcium gluconate (g/kg)
Processed olive 1 (Spain)	0.031 ± 0.00	ND
Processed olive 2 (Spain)	0.065 ± 0.007	ND
Processed olive 3 (Spain)	0.065 ± 0.004	ND
Processed olive 4 (Spain)	0.048 ± 0.003	ND
Processed olive 5 (Spain)	0.043 ± 0.003	ND
Processed olive 6 (Italy)	0.043 ± 0.013	ND
Processed olive 7 (Italy)	0.061 ± 0.013	ND
Processed olive 8 (Italy)	0.039 ± 0.007	ND
Processed olive 9 (Italy)	0.052 ± 0.001	ND
Processed olive 10 (Italy)	0.038 ± 0.003	ND
Beverage 1 (Korea)	ND	3.9 ± 0.0
Beverage 2 (Korea)	ND	3.8 ± 0.0
Beverage 3 (Korea)	ND	7.8 ± 0.5
Beverage 4 (Korea)	ND	3.8 ± 0.1
Beverage 5 (Korea)	ND	3.8 ± 0.0
Beverage 6 (Korea)	ND	3.8 ± 0.0
Beverage 7 (Korea)	ND	3.9 ± 0.1
Beverage 8 (Korea)	ND	5.7 ± 0.0
Beverage 9 (Korea)	ND	3.8 ± 0.0
Beverage 10 (Korea)	ND	3.8 ± 0.0

ND not detected

which were suitable for domestic use. To confirm the presence of FG and CG in samples, precursor and product ions were identified in the chromatograms of positive samples (*m/z* 447 and 411 for FG; *m/z* 431 and 333 for CG) (Table 5). In brief, the developed simultaneous LC–MS/MS method shows good applicability for FG and CG analysis in commercially processed olives and beverages.

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Availability of data and materials
All data generated or analyzed during the present study are included in this published article.

Declarations

Competing interests
The authors declare that they have no competing interests.

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