Analysis of Phytoestrogens in Soy Milk by GC/MS/MS with the Agilent 7000 Series Triple Quadrupole GC/MS

Application Note

Food

Authors

Imma Ferrer and E. Michael Thurman
Center for Environmental Mass Spectrometry,
University of Colorado, Boulder, CO

Melissa Churley, Harry Prest and Phil Stremple
Agilent Technologies, Inc
Santa Clara, CA

Phil Wylie and Jerry Zweigenbaum
Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808
USA

Abstract

An analytical method for the identification of eight plant phytoestrogens (biochanin A, coumestrol, daidzein, equol, formononetin, glycitein, genistein and prunetin) in soy milk was developed using gas chromatography tandem mass spectrometry (GC/MS/MS) with an Agilent 7000 Series Triple Quadrupole GC/MS. The phytoestrogens were extracted from soy milk with liquid-liquid extraction using ethyl acetate. The analytes were derivatized as their trimethylsilyl ethers with trimethylchlorosilane (TMCS) and N,O-*bis*(trimethylsilyl)trifluoroacetamide (BSTFA). The fragmentation patterns of the phytoestrogens were investigated by isolating and fragmenting the precursor ions in the collision cell (at different collision energies) and obtaining the corresponding product ion spectra. The general typical fragmentation for all the phytoestrogens studied involved the loss of a methyl and a carbonyl group. Two characteristic fragment ions for each analyte were chosen for identification, confirmation and quantitation. The developed methodology was applied to the identification, confirmation and quantitation of phytoestrogens in soy milk. Calibration curves were linear over 3 orders of magnitude.

Agilent Technologies

Introduction

Phytoestrogens are a group of nonsteroidal polyphenolic compounds that occur naturally in a wide range of plants and induce biological responses based on their ability to bind to estrogen receptors. Usually, they are present at high concentrations in legumes such as soy, clover, alfalfa, beans, and peas [1]. Beneficial effects of flavonoids (water-soluble plant pigments derived from the 2-phenyl-1,4-benzopyrone structure) on humans have been reported [2] and include their ability to scavenge free radicals. However, plant phytoestrogens have been also cited as possible endocrine disruptors in fish along with synthetic hormones and human estrogens from wastewater effluents [3]. Additionally, phytoestrogens, especially daidzein, genistein, and glycitein, have been correlated with low sperm counts in North American men with high and frequent soy food intake, more than twice per week [4]. Because phytoestrogens are excreted, not only by plants but also by humans and livestock via food consumption, it is important to evaluate their sources in food and their concentration in surface water and wastewater. A previous study from our group found important concentrations of daidzein and genistein in surface waters directly impacted by wastewater from a soy production plant [5].

Several analytical methodologies have been described for the identification and quantitation of flavonoids, including gas chromatography and liquid chromatography [6, 7]. Because of potential matrix interferences the use of selective ion-trap or tandem mass spectrometric methodologies (either LC/MS/MS or GC/MS/MS) is crucial to identify and confirm the presence of these analytes in complex samples, such as soy milk. In general, GC-based methods provide high resolution and low detection limits for the phytoestrogens, although they require derivatization to form the trimethylsilylether derivatives that increase volatility and improve thermal stability. Usually, MS/MS techniques that look at two or more characteristic fragments are required to fully characterize the silanized phytoestrogens since they provide higher selectivity than single ion monitoring techniques.

In this application, the Agilent 7000 Series Triple Quadrupole GC/MS instrument was used for the identification and confirmation of eight phytoestrogens in soy milk (Table 1). The phytoestrogens were identified using the characteristic fragmentation of the molecular ions for each of the analytes studied.

Experimental

Sample Preparation and Derivatization Procedure

Biochanin A, coumestrol, daidzein, equol, formononetin, genistein, glycitein and prunetin were purchased from Sigma-Aldrich (St. Louis, MO, USA). The derivatization reagents N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA), and trimethylchlorosilane (TMCS) were obtained from Sigma Aldrich (St. Louis, MO, USA). An aliquot of 500 µL of TMCS was added to 5 mL of BSTFA in order to have a 10% TMCS derivatization reagent. In the same way, 2 mL of pyridine were mixed with 8 mL of BSTFA to form a BSTFA/pyridine (5:1; v:v) solvent mixture for addition to the dry extracts before injection onto the GC. Methanol and ethyl acetate were obtained from Burdick and Jackson (Muskegon, MI, USA). All materials and reagents were of analytical purity. Standards of individual phytoestrogens were prepared in methanol at concentrations of 500 mg/L. From these solutions, diluted working solutions were prepared for derivatization and analysis by GC/MS/MS. All standard solutions were stored at –20°C and allowed to equilibrate at ambient temperature for at least 2 h before use.

The analytes were derivatized to their trimethylsilyl ethers with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS). The calibration standards were evaporated to dryness under a stream of nitrogen in silanized 5-mL reaction vials. Then, 200 µL of 10% TMCS/BSTFA derivatizing reagent was added to the vials and vortexed for 15 s. The vials were placed on a heating block at 60 °C for 1 h. After this time the vials were removed from the heating block, set aside 15 min to cool, and the reagents were evaporated to dryness under nitrogen. Finally, the dry residue was dissolved in 200 µL of 5:1 BSTFA/pyridine injection solvent and vortexed for 30 s. The derivatized extracts were transferred into autosampler vials using a clean silynized glass pipette and were analyzed by GC/MS/MS.

A simple and rapid procedure for the isolation of the phytoestrogens from soy milk was carried out using ethyl acetate as an extracting solvent. The soy milk sample (1 mL) was liquid-liquid extracted with 5 mL of ethyl acetate and evaporated to dryness under a stream of nitrogen. The residue was derivatized with TMCS and BSTFA/pyridine, at 60 °C for 1 h.

GC/MS/MS Instrumentation

The identification of phytoestrogens by GC/MS was carried out using an Agilent 7890 gas chromatograph coupled to a triple quadrupole mass spectrometer, Agilent 7000 Series Triple Quad GC/MS (Agilent, Santa Clara, CA, USA). The chromatographic separation was performed using an Agilent J&W HP-5 (5% phenyl, 95% methylpolysiloxane), 30 m × 0.25 mm id, fused-silica capillary column (Agilent Technologies, Santa Clara, CA, USA) of 0.25 µm film thickness. The carrier gas was helium at a constant flow rate of 1.2 mL/min held by electronic pressure control. Injector temperature was 280 °C, and splitless injection mode was used. The oven temperature pro-

gram was 100 °C (held for 1 min) to 240 °C at 40 °C/min (held for 1 min), to 300 at 10 °C/min (held for 4 min). The MS operating conditions were the following: positive electron ionization mode (EI+) using automatic gain control (AGC) with an electron energy of –70 eV. The ion source temperature was 300 °C. Gain voltage was set to 30. A dwell time of 50 msec was used for every MRM transition. One microliter of the extracts was injected on the system. MassHunter software was used for instrument control and data analysis.

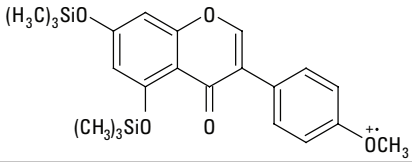
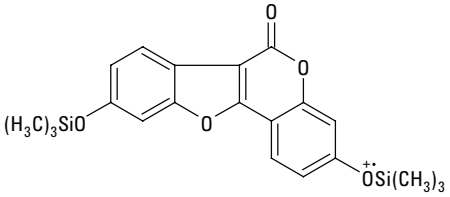
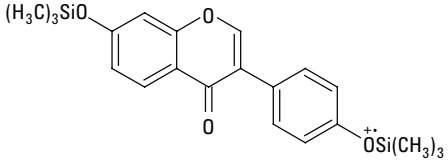
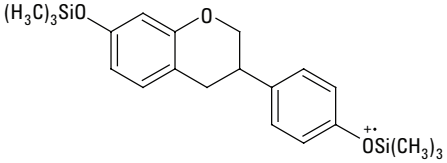
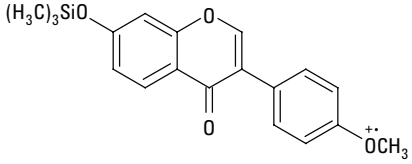
Results and Discussion

Optimization of GC/MS/MS Conditions

The initial study consisted of two parts. The first was to determine the precursor ion for each phytoestrogen in order to produce the largest signal. Typically the molecular ion was used for the precursor ion, except for biochanin A, genistein and prunetin, which gave the [$M^{+\bullet}$–$CH_3^{\bullet}$] as base peak ion (Table 1). Then, each compound was optimized again to determine collision energies for both the quantifying and qualifying ion transitions. Collision energies varied between 10 and 40 V. The optimized MRM transitions used for this study are shown in Table 2.

Table 2 shows the precursor ions and the main fragment ions for all the analytes studied. In some cases the fragmentation of the trimethylsilyl group is involved such as for genistein and prunetin. In other cases either a methyl or a methyl and a carbonyl group are lost and rearrangement of the structure

Table 1. Formula, Precursor Ion Chosen, and Chemical structures of the BSTFA Derivatives of the Phytoestrogens Studied

Name	Formula	M+ or M+•	Chemical structures
Biochanin A	$C_{22}H_{28}O_5Si_2^{+\bullet}$ *m/z* 428	413	
Coumestrol	$C_{21}H_{24}O_5Si_2^{+\bullet}$ *m/z* 412	412	
Daidzein	$C_{21}H_{26}O_4Si_2^{+\bullet}$ *m/z* 398	398	
Equol	$C_{21}H_{30}O_3Si_2^{+\bullet}$ *m/z* 386	386	
Formononetin	$C_{19}H_{20}O_4Si^{+\bullet}$ *m/z* 340	340	

Continued

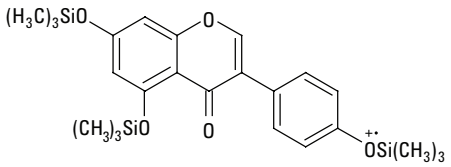
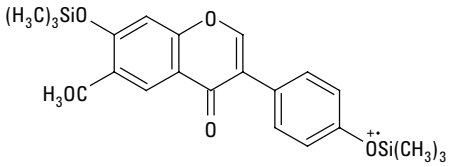
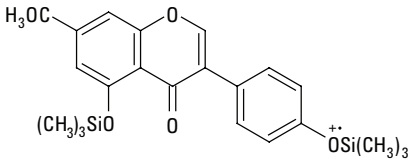
Name	Formula	M+ or M+•	Chemical structures
Genistein	$C_{24}H_{34}O_5Si_3^{+\bullet}$ *m/z* 486	471	
Glycitein	$C_{22}H_{28}O_5Si_2^{+\bullet}$ *m/z* 428	428	
Prunetin	$C_{22}H_{28}O_5Si_2^{+\bullet}$ *m/z* 428	413	

Table 2. MRM Transitions and MS Operating Parameters Selected for the Analysis of the Phytoestrogens Studied

Compound	MRM transitions (m/z)	Collision energy (eV)
Biochanin A	413 > 370	30
	413 >341	30
Coumestrol	412 > 397	20
	412 > 369	20
Daidzein	398 > 383	20
	398 > 355	30
Equol	386 > 207	10
	386 > 192	10
Formononetin	340 > 325	10
	340 > 297	20
Genistein	471 > 399	30
	471 > 327	40
Glycitein	428 > 413	10
	428 > 398	20
Prunetin	413 > 370	30
	413 > 341	30

occurs. For example, Figure 1 shows the detailed fragmentation pattern for daidzein. From an analytical point of view it is of primary importance to select those fragment ions that can be used for quantitative and confirmatory purposes and typically do not involve the loss of the TMS (trimethylsilyl) group. For this reason, two selective fragment ions were chosen when possible for each analyte, based upon the relative abundance. Only genistein incurred two losses of 72 mass units consistent with two losses of the TMS group and did not show the characteristic loss of a methyl radical and a CO group.

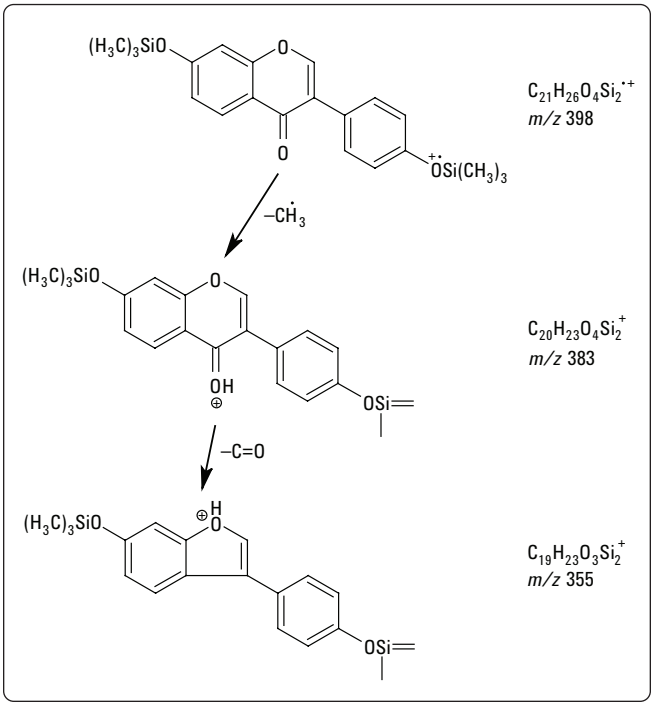


Figure 1. Fragmentation pathway for Daidzein.

Chromatographic Separation of the Phytoestrogens

GC/MS chromatographic conditions were optimized to achieve baseline resolution between all analytes while keeping an adequate run time of 16 min to maximize the throughput of samples. Because of the similar polarity exhibited by all of the derivatized phytoestrogens, the mix of compounds was separated using a slow temperature program (4 °C/min) in the GC/MS oven. Figure 2 shows an MRM chromatogram corresponding to a 50-ppb standard mix of all the phytoestrogens studied. Extracted ion chromatograms are overlaid for each one of the target analytes according to their respective quantifying MRM transition. Good chromatographic separation was obtained with the oven temperature program reported in the experimental section.

Analytical Performance

Calibration curves were plotted for concentrations of 0.1, 0.5, 1, 5, 10, 50 and 100 µg/L of standard solutions. The curves were found to be linear between 1 and 100 µg/L for all the analytes. An example is shown in Figure 3 for glycitein. Values for the coefficient of determination, R^2, were >0.99. Calculated instrumental limits of detection (LOD's) for all analytes are shown in Table 3. The lowest LOD's were for daidzein and prunetin. Formononetin showed the lowest sensitivity (that is, signal strength/concentration) and consequently produced a higher LOD. Intra- and interassay coefficients of variation (CV, n=3) for the standards ranged from 2 to 8%, showing good reproducibility of the methodology.

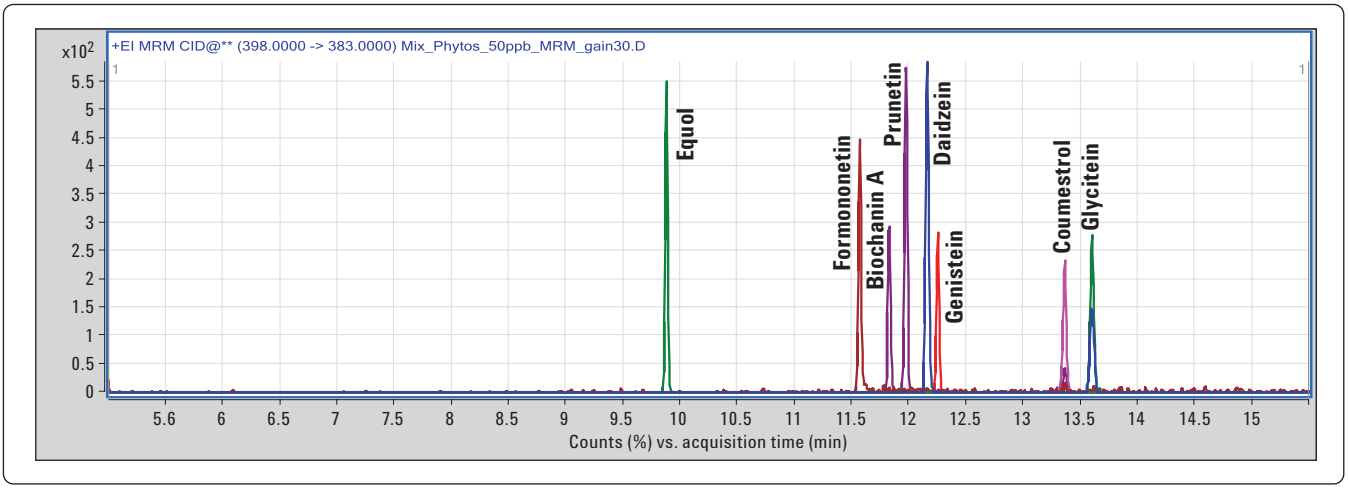


Figure 2. MRM extracted chromatogram for the eight phytoestrogens at 50 ppb concentration. Extracted ion chromatograms for the quantifier transition are shown.

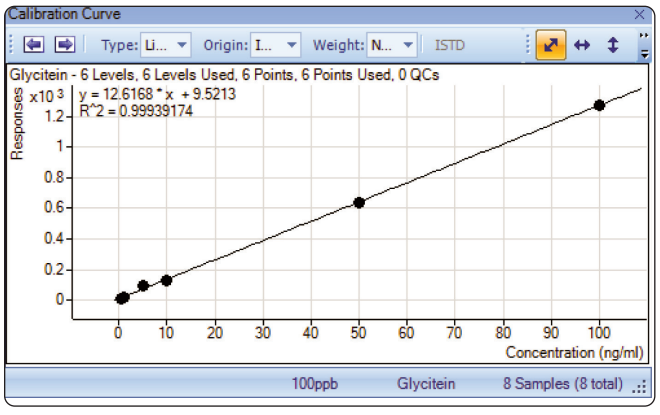


Figure 3. Calibration curve for glycitein using a seven point curve from 0.1 to 100 µg/L (ppb) using a linear fit with no origin treatment.

Table 3. Instrumental Limits of Detection (LOD's) for the Phytoestrogens Studied

Name	Calibration curve	R^2	LOD's (µg/L)
Biochanin A	21.8x – 25.1	0.992	2
Coumestrol	9.4x + 3.9	0.999	3
Daidzein	20x + 8.8	0.999	1
Equol	14.3x – 3.8	0.999	3
Formononetin	13.3x + 18.6	0.999	5
Genistein	11.4x – 12.5	0.994	2
Glycitein	12.6x + 9.5	0.999	2
Prunetin	41.7x – 59.3	0.992	1

Application to soy milk analysis

Figure 4 shows the chromatographic analysis of commercially available soy milk purchased from a local grocery store. Genistein (16,000 µg/L) and daidzein (7,000 µg/L) were the most common phytoestrogens identified in the soy milk sample. Also, the presence of glycitein at much lower concentration (760 µg/L) was confirmed by this method. Dilution of the extract had to be performed due to the high concentration of these two analytes in soy milk to avoid overloading of the signal in the detector. Figure 4 also shows the presence of the two MRM transitions for genistein and the respective ion ratios. As shown in this figure, phytoestrogens were easily identified in this complex matrix due to the selectivity of the MRM transitions and instrument sensitivity.

When running these types of complex samples on a GC/MS instrument it is very important to preserve the integrity of the system, especially the ion source and the chromatographic column. For this reason, a backflush procedure is recommended in these cases where the sample is complex and contains lots of interferents [8].

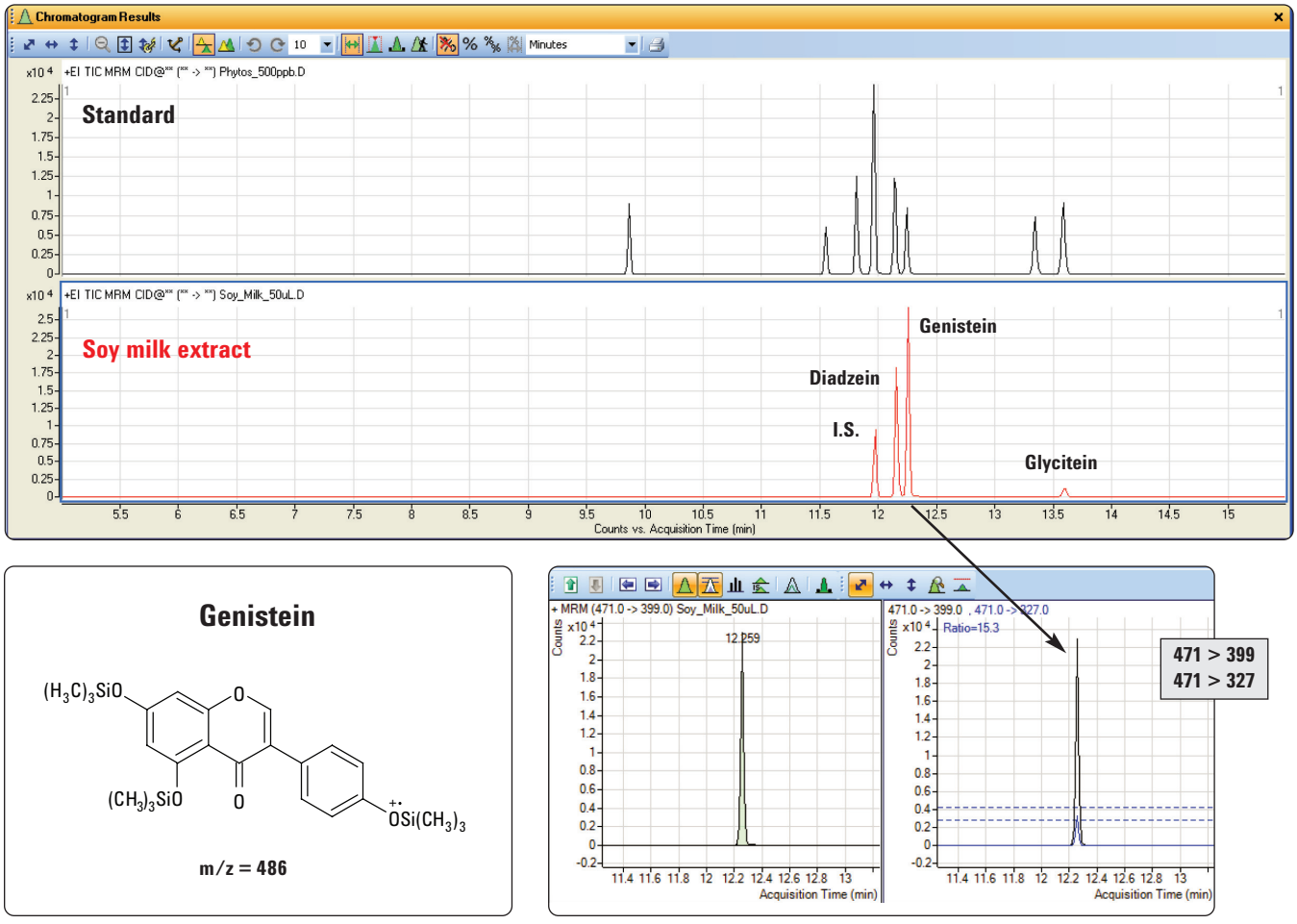


Figure 4. MRM chromatograms of a soy milk sample showing the identification of daidzein, genistein and glycitein. Ion ratios for the 2 MRM transitions for genistein are also shown.

Conclusions

An analytical method for the identification of eight plant phytoestrogens (biochanin A, coumestrol, daidzein, equol, formononetin, glycitein, genistein and prunetin) was developed using gas chromatography with tandem mass spectrometry (GC/MS/MS) using trimethylsilyl derivatives. The results of this study show that the Agilent 7000 Series Triple Quadrupole GC/MS is a robust, sensitive, and reliable instrument for the identification of phytoestrogens in soy milk samples. Furthermore, the fragmentation patterns of all the phytoestrogens were investigated by fragmenting the precursor ions in the collision cell. A typical fragmentation involving the loss of a methyl and a carbonyl group was discovered. Two characteristic fragment ions for each analyte were chosen for identification and confirmation. Finally, the developed methodology was applied to the identification and confirmation of phytoestrogens in soy milk.

References

1. K.R. Price, G.R. Fenwick, Food Addit. Contam. 2 (1985) 73.
2. J.J. Peterson, G.R. Beecher, S.A. Bhagwat, J.T. Dwyer, S.E. Gebhardt, D.B. Haytowitz, J.M. Holden, *J. Food Comp. Anal*. 19 (2006) S74.
3. M. Kawanishi, T. Takamura-Enya, R. Ermawati, C. Shimohara, M. Sakamoto, K. Matsukawa, T. Matsuda, T. Murahashi, S. Matsui, K. Wakamayashi, T. Watanabe, Y. Tashiro, T. Yagi, Environ. Sci. Technol. 38 (2004) 6424.
4. J.E. Chavarro, T.L. Toth, S.M. Sadio, R. Hauser, 23 (2008) 2584.
5. I. Ferrer, L.B. Barber, E.M. Thurman, *J. Chromatogr. A*, 1216 (2009) 6024.
6. S. Moors, M. Blaszkewicz, H.M. Bolt, G.H. Degen, Mol. Nutr. Food Res. 51 (2007) 787.
7. M. Erbs, C.C. Hoerger, N. Hartmann, T.D. Bucheli, *J. Agric. Food Chem.* 55 (2007) 8339.

8 C. Sandy, "GC-MS Method Robustness and Cycle Times Using Capillary Flow Technology and Backflushing," Agilent Technologies publication 5990-3367EN, 2009.

For More Information

For more information on our products and services, visit our Web site at www.agilent.com/chem.

www.agilent.com/chem

Printed in the USA
December 18, 2009
5990-5063EN

Agilent Technologies